CONTROL OF *SALMONELLA* BIOFILMS BY ESSENTIAL OILS AND REDUCTION OF *SALMONELLA* IN GROUND TURKEY BY LAURIC ARGINATE AND CARVACROL

By

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Salmonella is often associated with retail poultry products. Our research evaluated the effect of temperature on the biofilm formation by *Salmonella* spp. and the efficacy of essential oils in controlling these biofilms on stainless steel surfaces. The sublethal concentrations of thyme, oregano and carvacrol at 0.006-0.012% suppressed biofilm formation by *Salmonella* spp. while concentrations at 0.05-0.1% reduced the biofilms of a three-strain mixture of *Salmonella* spp. by 7 logs. Carvacrol was evaluated in combination with lauric arginate for controlling the three-strain mixture of *Salmonella* spp. in ground turkey containing 1%, 7% or 15% fat. Higher concentrations of carvacrol (1%) or lauric arginate (2000 ppm) when applied individually did not reduce *Salmonella* counts in ground turkey containing 7% fat. The combined mixture of carvacrol and lauric arginate at these higher concentrations was found to be synergistic in reducing the *Salmonella* counts by 4 log CFU/g in ground turkey containing 7% fat

DEDICATION

This work is dedicated to God Almighty for giving the grace to complete this work and to my lovely wife Laura Anne Oladunjoye for her support during my master's degree program.

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CHAPTER I

INTRODUCTION

Salmonella is one of the most important foodborne pathogens in the United States. Center for Disease Control (CDC) estimates that there are over 3.7 million cases of salmonellosis in the United States annually, costing about $3 billion (CDC, 2011). The outbreaks of salmonellosis have been frequently linked to retail poultry products. *Salmonella* persists in the form of biofilms on the food contact surfaces and in poultry processing environments which can become a source of contamination in processed retail poultry products. The critical control points identified in the poultry processing for the reduction of *Salmonella* are de-feathering steps. According to the Morbidity and Mortality Weekly Report (MMWR) 2010 report, three serotypes of *Salmonella* including, Enteritis, Typhimurium and Newport are most prevalent in foodborne diseases. Significant efforts are directed by the poultry industries towards *Salmonella* control during pre-harvest production and during post-harvest processing stages. Nevertheless continuous association of *Salmonella* in retail poultry products suggest improved strategies are needed for reducing *Salmonella* persistence at all stages of poultry production and processing (Bucher and others 2011).

Essential oils and recently FDA-approved lauric arginate are important antimicrobial agents for control of pathogenic microorganisms in food products. Essential oils are aromatic liquids derived from plants which contain terpenes, terpenoids, phenol-derived aromatic components, aliphatic components, aldehydes, ketones, acids and

isoflavonoids (Baik and others 2008). They are known for their broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative microorganisms (Hammer and others 1999; Dorman and Deans, 2000; Cutter, 2000; Delaquis and others 2002). However, there has been a very limited amount of work on optimizing the use of essential oils in food products (Burt, 2004).

In recent years, there have been several reports on the efficacy of plant essential oils against diverse Gram-positive and Gram-negative microflora by in-vitro assays (Karakaya and others 2011; Bajpai and others 2008; Lopez and others 2007). Nostro and others (2007) reported that essential oil of carvacrol at 1% resulted in a 4 log CFU/ml reduction of *S. aureus* or *S. epidermis* after 24 h exposure. Soković and others (2007) studied the antimicrobial activities of carvacrol oil against human pathogenic bacteria such as *Bacillus subtilis*, *Enterobacter cloacae*, *Escherichia coli* O157:H7, *Micrococcus flavus*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Salmonella enteritidis*, *Salmonella epidermidis*, *Salmonella Typhimurium*, and *Staphylococcus aureus*. Their study found that various essential oil components such as camphor, carvacrol, 1, 8-cineole, linalool, linalyl acetate, limonene, menthol, a-pinene, b-pinene, and thymol had higher antimicrobial activity as compared to all other components.

It is also hypothesized that at non-lethal concentrations, essential oils could interact with surface proteins of the bacterial cells and thereby reduces the bacterial cell attachment to the particular surface. Some active components such as thymol and carvacrol may penetrate through the polysaccharide matrix of the biofilms to cause the detachment of the biofilm cell mass. Currently, there are limited studies on the effects of essential oils against biofilms of *Salmonella* and their potential application in poultry products for controlling *Salmonella* prevalence (Barbosa and others 2009; Oussalah and

others 2007). Another antimicrobial agent of interest is the recently FDA approved Lauric arginate (LAE; ethyl N Lauroyl L-arginate Hydrochloride) (USFDA, 2005). Lauric arginate has a broad spectrum antimicrobial activity which is based on the disruption/instability of the plasma membrane lipid bilayer over a wide pH range (3-7).

Our research objectives were as follows: (a) to evaluate the effect of temperature on biofilm formation by *Salmonella* spp.; (b) to evaluate the effect of essential oils at sublethal and lethal concentrations on biofilms of *Salmonella* spp.; (c) to evaluate the effect of carvacrol and lauric arginate at different concentrations for controlling the three strain mixture of *Salmonella* spp. in ground turkey containing 1%, 7% or 15% fat; and (d) to evaluate the synergistic effect of carvacrol and lauric arginate in controlling the three strains mixture of *Salmonella* spp. in ground turkey.

CHAPTER II

LITERATURE REVIEW

2.1 *Salmonella* and its Pathogenicity

Salmonella is one of the most important foodborne pathogens and the cause of salmonellosis in susceptible individuals (Kendall and others 2003). Gastroenteritis and enteric fever are common clinical symptoms of these infections. *Salmonella* is ubiquitous in nature and it has often been isolated from food processing environments, processing water, farm animals and in foods such as poultry, meat, fruits and vegetables (Davies and others 1997; Dominguez and others 2002; Uyttendaele and others 1998; Huston and others 2002). Salmonellosis in humans is caused by ingestion of food products implicated with *Salmonella* contamination. *Salmonella* consists of over 2500 serotypes, which are mainly divided into three species namely, *Salmonella enterica*, *Salmonella bongori* and *Salmonella subterranean* (Su and Chiu, 2007; Agbaje and others 2011). In United States, only a limited number of *Salmonella* species are of major concern to public health. In particular, *Salmonella enterica* serovar Enteritidis and Typhimurium are most commonly associated with foodborne related incidences. According to the Center for Disease and Control (CDC), *Salmonella* causes about 45,000 cases of salmonellosis in humans and results in 400 deaths per year in United States (Isaacs and others 2005; Andrews-Polymenis and others 2010; Maurer and others 2011). The mode of infection by *Salmonella* is classified into two forms: invasive and non-invasive. The invasive form is responsible for severe illnesses such as cholecystitis and hepatobiliary carcinoma,

whereas the non-invasive results in a mild form of infection characterized by gastroenteritis and enteric fever (Penheiter and others 1997).

The systemic invasion of *Salmonella* into human host cells involves following stages: invasion, attachment/adherence, internalization and proliferation (Lawhon and others 2011). *Salmonella* Typhimurium possess highly efficient mechanisms of invasion; in the host body it escapes through stomach into the small intestine and invades the non-phagocytic M-cells in the distal ileum of the intestine to gain entry into the epithelium cells (Gunn, 2011; Bhowmick and others 2011). Initially, *Salmonella* invades and binds on the surface of the host epithelium cells by reversible and irreversible attachment with the help of fimbriae and type III secretion system T1. The type III section system is regulated by two regulatory systems: CpxR/CpxA and PhoP/PhoQ and the system also injects the virulence factors, also known as effectors, SopE, SopE2, SopB and SopA that triggers the polymerization of the actin which act as a mediator for the invasion of the epithelia cells (Raffatellu and others 2006; Layton and Galyov, 2007). In the epithelium cells, *Salmonella* cells replicates and disseminates within the microphages into the phagositic cell and are transferred into the mesenteric nymph nodes (Misselwitz and others 2011). After invasion, *Salmonella* is enclosed in a vacuole within the host cells in order to achieve maturation where it acquires endosome markers to position itself near the nucleus of the infected cells (Beuzon and others 2000; Bhavsar and others 2007).

After maturation in the vacuole *Salmonella* elicits the second set of virulence factors which are encoded on the *Salmonella* pathogenic islands (Fass and Groisman, 2009). These pathogenicity islands, namely SPI-1 and SPI-2 gets stimulated under two different responses and function at the site of the encoding type III secretion system that is required for the ability of *Salmonella* to stimulate cellular responses that are essential

for its pathogenicity (Eichelberg and Galan, 1999; Bhowmick and others 2011). The SPI-1 mediated invasion is dependent on the actin rearrangement that will promote engulfment of the bacteria subsequent to the release of the effectors (Clark and others 2011). While the SPI-2 type III secretion system plays a critical role in the systemic infection of *Salmonella*. The type III secretion systems in the pathogenicity islands are responsible for translocation of effectors into the host eukaryotic cells (Cardenal-Munoz and Ramos-Morales, 2011). These effectors facilitate in establishing the intracellular niches for *S*. Typhimurium in the host cell (Gunn, 2011). Subsequently, the pathogen sends a signal to the cells to induce a drastic membrane ruffling and cytoskeletal rearrangement which results in macropinocytosis and passive entry of the bacteria (Pieters, 2001; Lu and Goldberg, 2010). *Salmonella* interacts with the apical epithelia surface by means of cytotoxic effects that result in the destruction of M-cell and invasion of the enterocytes at both the apical and the basolateral phase (Finlay and Cossart, 1997). The infection spread through the host cell by a process known as paracytophagy into other organs of the body through the lymphatic system (Tezcan-Merdol and others 2001; Stevens and others 2006).

2.2 *Salmonella* transmission and its association with foodborne outbreaks

Salmonella is the second most frequent cause of foodborne related illnesses with a majority of the cases associated with consumption of contaminated food products such as poultry, meat, milk, seafood and fresh produce (Foley and Lynne, 2008). *Salmonella* is also associated with processed products such as peanut butter, infant formula, chocolate, cereal products, and dried milk (Podolak and others 2010; Strawn and others 2011). Currently, more than 2500 different *Salmonella* serotypes have been identified and

documented out of which only a few serotypes such as *S*. Typhimurium, *S*. Enteritidis, *S*. Newport, *S*. Heidelberg, and *S*. Montevideo are of significant interest, since 95% of human salmonellosis cases are associated with these serotypes (Foley and Lynne, 2008). It has been estimated that 95% of the non-typhoid *Salmonella* originating from the foods are of animal origin (Molbak, 2004; F and Wierup, 2006). Animal products, in particular poultry meats and eggs, are considered a primary source of *Salmonella* incidence (Duguid and North, 1991). Several sources have been identified from farm to fork for *Salmonella* transmission and its contamination in food products. In food processing environments, the contamination of a wide range of food products can occur due to poor handling, sanitary issues, poor equipment design, and poor ingredient control (Podolak and others 2010; Strawn and others 2011). At the farm level, contaminated feed is recognized as a primary source for the transfer of *Salmonella* into farm animals (Davies and others 2004). Also, the transfer of *Salmonella* from farm animals or grazing facility to the farmers, farm workers and their families as well as veterinarians serve as high risk factors for further dissemination of this pathogen (Molbak, 2004; Hoelzer and others 2011). *Salmonella* is also increasingly associated with fresh produce the use of contaminated water and animal manure as fertilizer in the farm practices are credited for the transfer of this pathogen to various fresh produce (Hanning and others 2009).

According to CDC estimates over the past five years, there are more than 25 incidences of *Salmonella* outbreaks, and several food products ranging from raw to ready-to-eat foods have been implicated (Brashears and others 2011; Friesema and others 2011). In April 2000, the consumption of turkey burger that was contaminated with *S*. Hadar infected 12 individuals in over 10 states. This particular *Salmonella* strain isolated from this multi-state outbreak was found to be resistant to clinical drugs such as

ampicillin, amoxicillin/clavulanate, cephalothin, and tetracycline (CDC 2011). More recently, in between the years 2009 and 2011, the multi-state *Salmonella* outbreak occurred in over 42 states with a total of 241 patients. These patients were reported to have come in contact with an African dwarf frog breeding facility in California. Notably, the most recent multi-state outbreak of a multi-drug resistant strain of *S. Heidelberg* in ground turkey resulted in one death and sickened 136 individuals in over 34 states. This outbreak led to the largest recall of 36 million pounds of ground turkey (CDC 2011).

2.3 Antibiotic resistance occurrence in *Salmonella* spp

The increasing emergence of antimicrobial resistance in *Salmonella* isolates due to use of antibiotics in farm animals is a major public health concern (van den Bogaard and Stobberingh, 2000; Gupta and others 2003; Plym and Wierup, 2006). It is quite common for *S. Typhimurium* to exhibiting multi-antimicrobial resistance to five antibiotics (Graziani and others 2008; Glenn and others 2011). Moreover, *Salmonella* serotype *Heidelberg* is also gaining serious attention due to its multidrug resistance and its increased association with foodborne outbreaks (Han and others 2011; Berrang and others 2009). Zhao and others (2008) analyzed ~10000 retail samples of ground turkey and chicken and identified 298 of these samples containing *S. Heidelberg*. Of these 298 isolates of *S. Heidelberg*, 49 isolates were resistant to five antimicrobials and eight were resistant to nine antimicrobials. In 2011, a multi-drug resistant strain of *S. Heidelberg* was associated with a multi-state outbreak of *Salmonella* leading to one of the largest meat recalls in the United States. The consumption of ground turkey meat contaminated with this multi-drug resistant *S. Heidelberg* infected 136 people in 34 states and one death (CDC 2011). The occurrence of antibiotic resistance in *Salmonella* has been

attributed to various factors such as the use of these antibiotics at sub-lethal concentrations, mass treatment and long term continuous usage of antibiotics (F and Wierup, 2006). The epidemiological, ecological, outbreak investigations and typing studies indicate that *Salmonella* spp. acquire their antibiotic resistance in the animals before their transmission to human by means of the food chain. One mode of transmission of antibiotic resistance in *Salmonella* strains is due to its association with commensal organisms that are capable of acquiring resistance genes from pathogenic microorganisms and such events are highly probable (Threlfall, 2002; Molbak, 2004).

In the United States, the emergence of fluoroquinolones antibiotic resistant *Salmonella* strains was first observed in the 1990s after its approval to treat poultry. The study conducted by national antimicrobial resistance system reveals the existence of a fluoroquinolone resistant strain of *S. Typhimurium* definitive phage type (DT) 104 in the United States (Glynn and others 1998). This strain of *Salmonella* is a multidrug resistant strain and is also known to be resistant to five other main antibiotics including ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracycline. In another study, the prevalence of *Salmonella* resistance strains was studied by the collection of 4008 *Salmonella* isolates from 51 states and about 97% (3903) of these isolates were serotyped; out of which 27% (1082) isolates were found to be resistant to four or more commonly used antibiotics such as ampicillin, chloramphenicol, streptomycin and sulfonamides. The results from this study also indicated that about 80% of these isolates were of multi-drug resistant strain of *S. Typhimurium* definitive phage type (DT) 104 (Herikstad and others 1997). In the United States, the outbreak related to multi-drug resistant *S. Typhimurium* DT104 strains was first observed in 1996 (Glynn and others 1998). The first multi-state outbreak of *S. Typhimurium* DT104 serotype occurred in the

United States in 2003 in which 58 cases were reported in over nine states. This outbreak was linked to the consumption of *Salmonella* contaminated ground beef (Dechet and others 2006). The *S. Typhimurium* DT104 strain was confirmed as an international multi-resistant clone (Threlfall, 2000). In 1993, this particular *Salmonella* strain was observed in Wales following the approval of fluoroquinolones in poultry. In addition, the prevalence of *Salmonella Typhimurium* definitive phage type (DT) 104 strain was evident in the year 2000 outbreak of *Salmonella* that infected 86 individuals in England which was linked to the consumption of contaminated milk from a dairy farm using fluoroquinolones (Walker and others 2000).

2.4 *Salmonella* biofilm formation in food processing environments

Biofilms are defined as heterogeneous communities of microorganisms adhering in a self-produced polymeric matrix on different surfaces in the food processing environments (Costerton and others 1999; Homoe and others 2009). Biofilms in the processing environment exists as either monospecies or as multi-species communities of microorganisms (Zottola and Sasahara, 1994). Previously, Knowles and others (2005) reported that a mixed species biofilm that included *S. Typhimurium* matures and reaches a quasi-steady state at which microenvironments inside these biofilm matrices are stable enough not to be affected by external selective pressure or nutrient depletion. And as a result of such microstructures, biofilms show high resistance to antimicrobial perturbation. Several pathogenic microorganisms such as *L. monocytogenes*, *Salmonella*, and *Campylobacter*, and several spoilage microorganisms such as *E. coli*, *Pseudomonas* and Lactic acid producing bacteria are capable of forming and residing within biofilm matrices in food processing environments (Kumar and Anand, 1998; Chmielewski and

Frank 2003). Biofilm formation involves following three distinct stages: (1) Adhesion, (2) Micro colony formation and (3) Maturation (Chmielewski and Frank 2003). Biofilm maturation is characterized by the production of a self-initiated extra cellular matrix (ECM) which consists of nucleic acids, protein or exopolysaccarides (EPS) (Crawford and others 2010). The EPS plays a critical role in imparting resistance to sanitizers, since it shields the bacterial cells from external stimuli such as nutrient availability, oxygen level and pH. This makes the eradication of biofilms difficult in comparison to their planktonic counterparts. Biofilm formation on a particular surface is governed by factors such as bacterial cell type, attachment surface, nutrient availability in the surrounding medium, background microflora, pH of the surrounding environment and temperature (Davey and O'Toole G, 2000). Moreover, the inherent bacterial cell factors such as flagella, pilli and surface appendages, proteins and polysaccharides assist in surface adherence (Crawford and others 2010). Bacterial attachment in biofilm formation is also influenced by factors such as type of the food matrix, physicochemical properties, hydrophobicity and auto-aggregation of the surface (Rosenberg and others 2008; Xu and others 2010; Van Houdt and Michiels, 2010).

One of the important reasons to study *Salmonella* spp. biofilm formation in the food processing environments is because of its ability to persist that leads to cross contamination and recontamination of food products. Several studies have suggested that the high prevalence of *Salmonella* in poultry processing environments. The moist conditions in the processing areas such as during evisceration and slaughter are ideal for *Salmonella* biofilm formation (Chmielewski and Frank 2003). The strong adherence of *Salmonella* biofilms in food processing environments and on the food contact areas is an important attribute for the persistence of this pathogen. For example, Vetsby and others

(2009) reported that persistence of various *Salmonella* isolates in fish and feed factories depends on the biofilm forming capabilities of these strains. *Salmonella* cells present in the biofilm matrix show a much higher resistance compared to their planktonic counterpart against routinely used cleansing reagents (Bridier and others 2011; Joseph and others 2001). Reasons for this resistance are due to the phenotypic aggregation of the microbes and presence of exopolysaccharides that slows the diffusion of these antimicrobial agents which ultimately result in microbial cells being exposed to sublethal concentration of various biocides (Allison and others 1998; Bridier and others 2011). Similar to other foodborne pathogens, *Salmonella* possess the ability to form biofilms on a wide variety of surfaces including plastic, metal, glass, rubber surfaces. Kroupitski and others (2009) reported noticed that *Salmonella* isolates that show better biofilm formation by in-vitro screening also result in a preferentially stronger attachment to the cut lettuce surfaces and such cells were more tolerant to a lethal acidic treatment when compared to their planktonic counterparts. Rodrigues and others (2011) reported that *S. Enteritidis* is able to colonize processing surfaces such as granite, marble, stainless steel, and siltstones during the adhesion and biofilm formation process. In addition to their surface properties and continuous residual nutrient availability on the food processing surfaces, research studies have suggested that the certain changes occurring at the genomic level assist in *Salmonella* biofilm formation. Quorum sensing which involves cell to cell communication and controls expression of genes and proteins expression is also one of the factors that is responsible for *Salmonella* biofilm formation (Li and others 2002).

2.5 Approaches for control of *Salmonella* biofilms in food processing environments

The biofilm phase protects the bacterial cells from environmental stimuli such as chemical sanitizers that are commonly employed in the food processing environments. Presence of organic residue, water temperature and water hardness as well as the nature of the finished surface limits the sanitizing efficacy of these chemical agents against *Salmonella* biofilms (Marin and others 2009). Some of the chemical agents frequently used for eliminating *Salmonella* biofilms in food processing environments include: sodium hypochlorite, sodium chlorite, potassium chlorite, quaternary ammonium, phenol, cresol, Tween-20, NaOCl, acetic acids, Na2PO4, H2O2, Al-acide, Oxine, Carnebon, and enzymes. Previous studies have indicated that treatment of *Salmonella* biofilms with the above chemical agents resulted in approximately 2-3 log reductions of *Salmonella* biofilm cells (Yu and others 2001; Han and others 2004; Stringfellow and others 2009). The age of the biofilm also plays a critical role in the eradication of the biofilms (Yang and others 2009). Wong and others (2010) examined the susceptibility of disinfectants such as benzalkonium chloride, chlorhexidine gluconate, citric acid, quaternary ammonium compounds, sodium hypochlorite (SH) and ethanol against 3-day-old biofilm and planktonic cells of *Salmonella*. Results from the study suggested that the disinfectants were more effective against planktonic cells in comparison to the 3-day old biofilm cells. In another study, the antimicrobial efficacy of sanitizers and detergents such as benzalkonium chloride, alkyldiaminoethyl glycine hydrochloride, chlorhexidine digluconate, and polyhexamethylenebiganide were found to be effective against *S. Enteritidis* biofilms but not against biofilms of *E. coli* and *S. aureus* (Ueda and Kuwabara, 2007). Moreover, Hasegawa and others (2011) tested the efficacy of lethal treatments of HCl, acetic acid and rice vinegar on *S. enterica* isolates that had varying

biofilm formation capabilities and found no differences in survival of biofilm cells of these isolates with HCl while acetic acid and vinegar showed better eradication capability

2.6 Prevalence of *Salmonella spp.* in turkey processing environments

Prevalence of *Salmonella* in ground turkey, chicken and beef products is a major concern to the meat processing industries (Dincer and Baysal, 2004). Poultry breeding and processing has been associated with *Salmonella* persistence and consequently this pathogen has also been associated with various poultry products including turkey carcasses and ground turkey (McPherson and others 2006; Snow and others 2011; Aury and others 2010; Reynolds and others 2010). In 2009, the CDC reported 7039 laboratory confirmed cases of *Salmonella*. In addition, serotyping of this laboratory collected isolates from these cases suggested that *Enteritidis*, *Typhimurium*, and *Newport* were most prevalent and that these three serotypes accounted for 19.2%, 16.1% and 12.1% prevalence, respectively (MMWR, 2010). A prevalence study conducted by Arslan and Eyi (2010) indicated that out of 225 meat samples analyzed from poultry, ground beef, and beef samples, about 22% of these samples were tested positive for *Salmonella* contamination. The *Salmonella* strains that were most frequently isolated in this study were *S. Typhimurium*, *S. bongori* and *S. enterica*. Moreover, Iseri and Erol (2010) studied the prevalence of *Salmonella* in packaged retail ground turkey. In the study, 240 samples of ground turkey were examined for *Salmonella* contamination and the results indicated that 45% of the samples tested positive for *Salmonella*. Prevalence of *Salmonella* in food processing plant has also been investigated to find out the critical areas of contamination during processing steps. The results from several studies have revealed that defeathering and pre- and post-chilling processing steps had the highest incidence of *Salmonella*

contamination (Whyte and others 2001; Logue and others 2003; Nde and others 2006). Considering the high prevalence of *Salmonella* and their wide spread resistance to antimicrobials, this necessitates the use of more stringent control measures for prevention of *Salmonella* contamination in turkey and other meat processing environments

2.7 Intervention strategies for control of *Salmonella* in turkey processing environments and in ground turkey

Poultry birds are a potential source for the dissemination of *Salmonella* in food processing. Therefore, significant efforts are directed towards identifying the control measures during the pre-harvest production stage. Much of this effort has led to better hygiene practices such as proper handling, storage, incorporation of HACCP, effective sanitization of breeding facilities and continuous monitoring for *Salmonella* prevalence (Vadhanasin and others 2004). Also, use of probiotic cultures during feeding of chicks and turkey poults resulted in about 2 log CFU/g reductions of *S*. Heidelberg counts when compared to the control samples that contained 3 log CFU/g of *S*. Heidelberg (Menconi and others 2011). Similarly, other studies also demonstrate the effectiveness of different probiotic organisms for *Salmonella* reductions in broilers (Higgins and others 2007; Vicente and others 2008). Incorporation of small chain fatty acids such as butyric acid sodium salt have also been effective at reducing *S*. Enteritidis in broiler chickens (Fernandez-Rubio and others 2009). Nevertheless, continuous association of *Salmonella* with retail poultry products suggest that even though pre-harvest strategies can aid in reducing *Salmonella* persistence, additional control measures against this pathogen are very important during postharvest processing (Bucher and others 2011)

Currently, several strategies are being used to control *Salmonella* in poultry and meat processing industries. A variety of antimicrobial rinses such as acid wash (Laury

and others 2009), acidic calcium sulfate (Benli and others 2011) and electrolyzed oxidizing water (Fabrizio and others 2002) have been evaluated for use in poultry products. In a study conducted by Dow and others (2011) a *Lactobacillus*-based intervention comprising of NP51, NP35, NP3, and NP7 at 106 CFU/cm2 yielded reduction of *Salmonella* in ground turkey by 2 log at 5°C after 24 h. Study conducted by Keklik and others (2010) showed that the exposure of ultra violet rays to chicken breast for 30 s prior to vacuum-packaging resulted in ~2 log reduction of *Salmonella*. However, irradiation treatment while effective in reducing microbial load from chicken breast also caused lower texture and flavor attributes during 14 day refrigerated storage (Lewis and others 2002). The study conducted by Stringfellow and others (2009) revealed that reduction of organic matter on poultry carcass leads to 2-4 log reduction of *S.* Typhimurium by quaternary ammonium compounds, binary or chlorhexidine. Recent interest in the broad antimicrobial activity of essential oils may spur their potential applications in various food systems and may lead to their effective use in controlling the growth of *S.* Typhimurium in poultry products and poultry processing environments.

2.8 Essential oils and their diversity from various plant extracts

Essential oils are liquid, volatile compounds that are characterized by pungent smells and derived from different parts of aromatic plants parts such as flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots (Burt, 2004; Solorzano-Santos and Miranda-Novales, 2011). Essential oils are stored as secondary metabolites in plant organs and are commercially extracted using steam hydro-distillation (Bakkali and others 2008). Essential oils that are derived from various plant extracts are alternative biocides that have recently gained interest in research since chemical reagents that are currently

used are susceptible to resistance development. Essential oils have a diverse range of properties such as antibacterial, anti-fungal and antiviral properties (Bakkali and others 2008). Carvacrol and thymol are active component of essential oil that has an additive effect against S. aureus and P. aeruginosa (Lambert and others 2001). The essential oil of oregano used in combination with sodium nitrate inhibited the toxin production by C. botulinum in a broth model (Ismaiel and Pierson 1990). There was a loss of antibacterial activity in rice when salt at 1.25 g/l was used in combination with carvacrol and p-cymene against B. cereus (Ultee and Smid, 2001). The essential oil components which are mainly responsible for their antimicrobial properties include terpenes, terpenoids, phenol-derived aromatic components, aliphatic components, aldehydes, ketones, acids and isoflavonoids (Baik and others 2008). The varying composition of these phenolic components is responsible for their antimicrobial activities and their mode of action (van Vuuren, 2008; Tiwari and others 2009). In addition, factors such as plant maturity and method of extraction of essential oils also influence the antimicrobial properties of essential oils (Huie, 2002). Essential oils are not readily soluble in water and so additional solvents and emulsifiers are needed to enhance their solubility. Their solubility can be enhanced using solvents such as ethanol, methanol, tween-20 and tween-80, acetone in combination with tween-80, polyethylene glycol, propylene glycol, n-hexane, dimethyl sulfoxide and agar (Burt, 2004).

2.9 Mode of action of essential oils

Antimicrobial efficacy of essential oils and their mode of action have been examined over a wide variety of organisms including both Gram-positive and Gram-negative microorganisms. Essential oils penetrate the cell wall and cytoplasmic

membrane to disrupt the structure of the polysaccharides and phospholipids which results in increased permeability of the membrane. The permeability is characterized by the loss of ions such as the K+ ion, collapse of the proton pump and depletion of the ATP pool (Hong and others 2004; Di Pasqua and others 2006; Di Pasqua and others 2007). Furthermore, essential oils can coagulate the cytoplasm, and damage the protein, the fatty acid, the cell wall and eventually cause lysis of the cell which results into death of the bacterial cell (Lambert and others 2001; Oussalah and others 2006). Antimicrobial efficacy of essential oils depends upon the concentration of their phenolic constituents. When the concentration of phenolic components is low, it affects the enzyme activity of the bacterial cell, and when the concentration is high, it causes protein denaturation (Tiwari and others 2009). The presence of OH-group in chemical structure of the phenolic components of essential oils such as carvacrol and thymol is also responsible for their antimicrobial and antioxidant properties (Ultee and others 2000; Dorman and Deans, 2000). The OH group influences the essential oils components effectiveness by ensuring even distribution of the antimicrobial properties within the aqueous and non-aqueous state and reducing bacteria selectivity (Dorman and others, 2000).

2.10 Antimicrobial activity of essential oils against biofilms of foodborne pathogens

The ability of various *Salmonella* enterica serotypes to form resilient biofilms on a wide range of surfaces coupled with the increasing resistance of these organisms to conventionally used chemicals has prompted the use of natural antimicrobials. Essential oils constitute a mixture of several active antimicrobial compounds such as thymol, carvacrol, p-cymene and γ-turpinine and are believed to reduce the chance of resistance development in bacterial cells (Daferera and others 2003). Some chemical agents cause

corrosion of the contact surfaces as well as toxicity effects that have led to interest in the use of natural antimicrobials for the control of biofilms (Knowles and Roller, 2001). Several studies have suggested that essential oils of thyme, oregano and carvacrol are effective at reducing and eradicating foodborne pathogens and their corresponding biofilms (Ultee and others 1999; Knowles and Roller, 2001; Simoes and others 2009). For example, carvacrol at 1% resulted in a 4 log CFU/ml reduction of S. aureus or S. epidermis after 24 h exposure. Essential oils at non-lethal concentration could interact with the surface proteins of bacterial cells and thereby reduce the bacterial cell attachment to that particular surface (Nostro and others 2007). Another possible mechanism could be reduced motility and flagella production in the presence of these essential oils as previous studies demonstrated that the presence of carvacrol at non-biocidal concentrations reduced flagella synthesis and motility in E. coli O157:H7 cells (Burt and others 2007). Such inhibition of the bacterial biofilm formation is also observed with the ground beef derived small chain fatty acid on E. coli K-12 cells (Soni and others 2008) and by citrus flavonoids and limonoids on E. coli O157:H7 and V. harveyi cells (Vikram and others 2010; Vikram and others 2011).

At lethal concentrations, essential oil biocides are proposed to interact with the lipid bi-layer of cytoplasmic membranes which results into damage and loss integrity of the cell membrane with subsequent leakage of the cellular material from inside the cells (Ultee and others 1999; Burt, 2004). Moreover, active essential oil components such as thymol and carvacrol due to their hydrophilic properties and strong antimicrobial activity are theorized to penetrate through the polysaccharide membrane of the biofilm matrices and such diffusion causes the detachment of the biofilm cell mass (Nostro and others 2007). in addition Nostrol and others (2007) demonstrated that biofilm inhibitory

concentration for S.aureus and S.epidimis was 0.125–0.5% for oregano and 0.031–0.125% for both carvacrol and thyme whereas the biofilm eradication concentration was 0.25–1.0% for oregano and 0.125–0.5% for the essential oil of carvacrol and thyme and these values were 2-4 fold greater than the concentration required to inhibit their planktonic counterparts.

2.11 Antimicrobial activity of essential oils in food systems

Application of essential oils in food systems has been limited because essential oils are known to impart strong flavor and aroma to food products. However, essential oils when used in combination with other hurdle technologies can reduce the concentration of essential oils required and in turn reduce their organoleptic challenges (Burt, 2004). Factors such as complexity of the food system, storage temperature and pH will determine the concentration of essential oils that are needed in the final product composition (Tassou and others 1995; Gill and others 2002). The amount of essential oils that are required in food substrate is generally 10- 100 fold higher than that required in broth (Burt, 2004). The antimicrobial activity of essential oils have been tested over a wide range of food products such as such as meat, fish, milk, dairy products, vegetables, fruit, and cooked rice (Burt, 2004). The application of 5% rosemary essential oil gave < 3 log CFU/g reduction of *Listeria monocytogenes* in pork liver sausage (Pandit and Shelef 1994). Antimicrobial coating of shrimp with 0.75-1.5 % thyme and cinammaldehyde essential oil resulted in a 1.5 log reduction of Pseudomonas putida (Ouattara and others 2001). Mendoza-Yepes and others (1997) tested the antimicrobial efficacy of preservatives formulated with 50% essential oils of rosemary, sage and citrus in soft cheese at final concentrations of 250-2500 ppm which yielded a 1.5 log CFU/g reductions

against L. monocytogenes. In a similar study, the application of clove essential oil at 0.5-1.0% in mozzarella cheese resulted in a 1.5 log CFU/g reduction of L. monocytogenes (Vrinda and Garg 2001). Furthermore, the antimicrobial studies have also been tested on fruits and vegetables. For example, rinsing of lettuce and carrots with a solution containing 0.1-10 ml/l of thyme essential oil showed a 1.5 log reduction against E. coli O157:H7 on lettuce and 1.5-3.0 log reductions in carrots (Singh and others 2002). In another study, farm produce such as grapes and tomatoes were washed with essential oil solutions of thymol and carvacrol at 0.4 mg/ml against *Salmonella*. Results from this indicated >4.1 log reductions of S. Typhimurium and *S.* kentucky after 5 min of washing (Lu and Wu, 2010). Currently, there is no published information on the antimicrobial efficacy of essential oils against S. Typhimurium in ground turkey products.

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CHAPTER III

REDUCTION OF BIOFILMS OF *SALMONELLA* TYPHIMURIUM AND *SALMONELLA* ENTERICA ON POLYSTYERENE AND STAINLESS STEEL SURFACES BY ESSENTIAL OILS

3.1 Introduction

Salmonella is a prevalent foodborne pathogen and the cause of salmonellosis in susceptible individuals (Kendall and others 2003), and gastroenteritis and enteric fever are common clinical symptoms of these infections. *Salmonella* is ubiquitous in nature, has often been isolated from food processing environments, processing water, farm animals and foods such as poultry, meat, fruits and vegetables (Davies and others 1997; Dominguez and others 2002; Uyttendaele and others 1998; Huston and others 2002). The ability of *Salmonella* to form biofilms is a primary concern in food processing environments. The adherence of *Salmonella* cells as biofilms in the processing environment and in food products is an important attribute that contributes to the persistence of this pathogen. Vestby and others (2009) reported that the persistence of various *Salmonella* isolates in fish and feed factories depends on the biofilm forming capabilities of these strains. Better biofilm formation correlates to higher persistence. Kroupitski and others (2009) noticed that those *Salmonella* isolates which show better biofilm formation by in-vitro screening also resulted in a preferentially stronger attachment to cut lettuce surfaces, such cells were more tolerant to a lethal acidic treatment when compared to their planktonic cells. In addition, Rodrigues and others

(2011) showed that S. enteritidis is able to colonize processing surfaces such as granite, marble, stainless steel, and siltstones during adhesion and the biofilm formation process. *Salmonella* cells present in the biofilm matrix show higher resistance than their planktonic counterparts against routinely used cleansing reagents (Bridier and others 2011; Joseph and others 2001). The reasons for this resistance are to the phenotypic aggregation of the bacterial cells and the presence of exopolysaccarides that slow the diffusion of these antimicrobial agents; which ultimately results in these microbial cells being exposed to the sub lethal concentration of various biocides (Allison and others 1998; Bridier and others 2011). Sodium hypochlorite, acidic and alkaline detergent solutions, benzalkonium chloride, quaternary ammonium, ethanol, iodophor and gluconate are some of the chemical interventions evaluated against *Salmonella* biofilm cells (Wong and others 2000; Rodrigues and others 2011a; Ueda and Kuwabara, 2007; Joseph and others 2001). Though the degree of biofilm inactivation varies mainly depending on the concentration and exposure time of these chemicals (Wong and others 2000), sodium hypochlorite in general appears to have better inactivation against *Salmonella* biofilm cells (Rodrigues and others 2011a). In another report, the use of 200 ppm active chlorine concentrations from sodium hypochlorite did not show complete inactivation of *Salmonella* biofilm cells (Ueda and Kuwabara, 2007). Also, exposure of *Salmonella* biofilm cells to either sodium hypochlorite or benzalkonium chloride induced expression of stress protective genes in surviving cells (Mangalappalli-Illathu and Korber, 2006; Rodrigues and others 2011a).

Essential oils derived from plant materials are alternative biocides that have recently gained attention since many currently used chemical reagents are becoming prone to resistance development by the target species. Moreover, each essential oil

constitutes a mixture of several active antimicrobial compounds such as thymol, carvacrol, p-cymene and γ-turpinine, which reduces the chances of resistance development in bacterial cells (Daferera and others 2003). In recent years, there have been several reports on the efficacy of plant essential oils of different origins against diverse Gram positive and Gram negative microflora using in vitro assays (Karakaya and others 2011; Bajpai and others 2008; Lopez and others 2007).

Currently, there has been limited work on determining the efficacy of these essential oils in various food substrates and against environmentally relevant biofilm cell masses in particular for *Salmonella* isolates (Barbosa and others 2009; Oussalah and others 2007). This study was conducted to: (a) to determine the effect of temperature on *Salmonella* biofilm formation; (b) evaluate the antimicrobial efficacy of various plant essential oils against different *Salmonella* strains; (c) determine the efficacy of thyme, oregano and their active antimicrobial constituent carvacrol in inhibiting *Salmonella* biofilm formation at non-biocidal concentrations; and (d) determine the exposure time and concentration dependent efficacy of selected essential oils at biocidal concentrations against individual and mixed strains of *Salmonella* cells that were present in either planktonic or as a biofilm cell mass on polystyrene and stainless steel surfaces.

3.2 Materials and Methods

3.2.1 *Salmonella* culture preparation

Three strains of *Salmonella* spp. were used. These were S. Typhimurium ATCC-14028, S. Typhimurium ATCC-19585, and S. enterica ATCC-23564. These stock cultures were maintained in tryptic soy broth (TSB) slants at 4°C and a working culture of these *Salmonella* strains were prepared by inoculating 10 µl of the stock solution into

10 ml TSB and incubating at 37°C for 18 h to obtain a cell concentration of ~109 CFU/ml. To obtain a mixed cell suspension of these *Salmonella* strains, 1 ml of overnight grown cell suspensions of each of these three strains were added into 7 ml of TSB. The mixture was serially diluted in 0.8% saline to achieve the desired cell concentrations.

3.2.2 Antimicrobials

The essential oils of thyme, cinnamon leaf, eugenol, linalool, limonene, carvacrol and bay used in these experiments were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, MO, United States); lemon, orange and oregano essential oils were purchased from Now Foods Inc. (Nowfoods, Bloomingdale, IL, USA). Based on the manufacturer's specifications, all essential oils had ≥ 98% purity. For experimental purpose, the essential oils were initially solubilized by mixing with an equal volume of propylene glycol (PG) (50:50).

3.2.3 Agar disk-diffusion assay

The nine essential oils were screened for their antimicrobial efficacy using a disk-diffusion assay against three different *Salmonella* strains namely S. Typhimurium ATCC-14028, S. Typhimurium ATCC-19585, and S. enterica ATCC-23564 on the agar surface. An overnight culture of each *Salmonella* strain was grown in tryptic soy broth (TSB) to achieve an inoculum level of ~ 109 CFU/ ml and further dilutions were made in 0.8% saline to achieve an inoculum level of ~ 107 CFU/ml out of which 100 µl of *Salmonella* cell suspensions were spread plated on XLD and tryptic soy agar (TSA). A sterile filter paper disc (6 mm in dia.) was placed aseptically in the center of the agar plates and subsequently soaked with 10 µl of different essential oils. The TSA plates were incubated at 37°C for 24 h and XLD plates were incubated at 37°C for 48 h. After the incubation

period, the diameters of the zones of inhibition were measured and recorded in (mm) using a ruler. This experiment was repeated three times.

3.2.4 Minimum Inhibitory and Minimum Bactericidal Assay

The essential oils of thyme, oregano and carvacrol were evaluated for their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) against all three *Salmonella* strains (S. Typhimurium ATCC-14028, S. Typhimurium ATCC-19585, and S. enterica ATCC-23564). Two-fold dilutions of each of the essential oils were prepared in PG and 100 µl of these diluents were added into 50 ml of TSB to achieve the desired essential oil concentrations ranging from 0.1 % to 0.006%. These TSB solutions containing different essential oil concentrations were placed in 24-well microtiter plates and duplicate wells were inoculated with each of the *Salmonella* strains using 10 µl from the overnight grown cultures to achieve a bacterial cell concentration of ~ 107 CFU/ ml. The plates were incubated at 37°C for 24 h. The MIC was noted as the lowest essential oil concentration showing no visible *Salmonella* growth in TSB. The MBC was examined by spread plating 250 µl of the content from the wells that did not show visible growth in the presence of essential oils in TSB. The plates were incubated at 37°C for 24 h and the minimum essential oil concentrations that did not show visual colony formation were reported as the MBC. This experiment was repeated three times.

3.2.5 Effect of temperature on biofilm formation by *Salmonella* spp. in polystyrene 24-well microtiter plates

An overnight culture of each of the three *Salmonella* strains was inoculated using 10 μl of stock solution into triplicate wells of 24-well microtiter plates that were filled

with 2 ml TSB which resulted in an initial inoculum level of ~ 10^7 CFU/ ml in each well. A total of 3 plates were prepared and one plate of each was incubated at temperatures of 22°C (room temperature), 30°C and 37°C for 24 h. Following the incubation period, the TSB growth medium that contained planktonic cells was removed and individual wells were washed to remove loosely bound cells from the well surfaces. This washing step included filling of individual wells with 2 ml of sterile DI water and waiting for 2 min before pipetting out, which was repeated 3 times and then the biofilm mass adhered to the well surface was quantified.

To quantify the firmly attached biofilm mass on the well surfaces, each well was filled with 2 ml of 1% crystal violet solution and plates were incubated at room temperature for 15 min. After this staining step, the crystal violet solution from each well was removed and the wells were further washed five times with sterile DI water as described above to remove any residual crystal violet. Subsequently, the bound crystal violet stain that was bound to the well surface was solubilized using solution 2 ml/well of ethyl acetate (80% ethanol and 20% acetone) solution. The OD of each well was measured at OD_{562}nm using an automated microtiter plate reader (Bio-Tek, model-ELX800NB).

3.2.6 Effect of sublethal concentrations of essential oils on *Salmonella* biofilm formation

The purpose of this assay was to determine if sublethal concentrations (≤ 1/2 MIC) of thyme, oregano and carvacrol essential oils can inhibit the ability of *Salmonella* spp. to produce biofilm in the 24-well polystyrene microtiter well plates. These essential oils were serially 2-fold diluted in PG and 500 µl from the appropriate dilution tube was added into 25 ml of TSB to yield sublethal essential oil concentrations of 0.006% and

0.012%. The control treatment did not contain any essential oils. These TSB solutions containing sublethal concentrations of essential oils were distributed into 24-well microtiter plates (2 ml/well) and triplicate wells were inoculated with 10 µl of the overnight grown culture of each of the three *Salmonella* strains. The microtiter plates were incubated at 22°C for 24 h and turbidity measurements (OD630nm) were taken with a microtiter plate reader (Bio-Tek, model- ELX800NB) to ensure that there were no differences in growth. Following this step, planktonic and loosely attached cells were removed by washing the individual wells three times using sterile DI water. Subsequently, microtiter plates were processed for the quantification of biofilm mass using crystal violet staining procedure that was described above.

3.2.7 Effect of sublethal and lethal concentrations of essential oils on the reduction of preformed biofilms of *Salmonella*

This assay was conducted to determine if the essential oils of thyme, oregano and carvacrol possess the ability to eradicate *Salmonella* cells that are present as biofilm mass. For this purpose, 24-well microtiter plates that are filled with 2 ml/well TSB was inoculated with 10 µl of overnight grown cultures of different *Salmonella* strains. The plates were incubated at 22°C for 24 h in a static condition for biofilm production. After incubation, the planktonic cells and loosely attached cells from the well surface were removed by washing each well with sterile DI water 3 times. Subsequently, the biofilm cells attached to the well surface were treated with essential oil solutions by adding 2 ml/well of TSB containing thyme, oregano or carvacrol at 0% (control), 0.012%, 0.025%, 0.05% and 0.1%. Preparation of these TSB solutions contained essential oil concentrations that were determined by MIC/MBC assay section. Six wells were used for each concentration of the essential oil type and *Salmonella* strain, and plates were

incubated at 22°C for 24 h. After the incubation period, TSB media from the individual wells was removed and each well was washed three times using sterile DI water. Subsequently, plates were processed for measurement of any remaining biofilm cell mass using crystal violet staining assay. The procedure for crystal violet staining of the biofilm mass and its quantification at OD562nm was described above.

3.2.8 Time dependent kill of *Salmonella* biofilms by essential oils in a 24-well polystyrene well surface

A three strain mixture of *Salmonella* biofilms was produced in 24-well microtiter plates and treated with essential oils of thyme, oregano and carvacrol at varying concentrations as described above in section 3.2.6. In this assay, the biofilm was exposed to essential oil treatments for 1 h, 4 h or 24 h; following this time period, the plates were processed to determine the effect of exposure time on reductions in in *Salmonella* biofilms upon treatment with different essential oils at varying concentrations. Reductions in biofilm cell mass was performed based on CFU counts (Sillankorva and others 2008). For this purpose, each of the triplicate wells was first filled with 2 ml of peptone water (containing 0.1% peptone and 0.02% Tween-80) and the biofilm mass attached on the surface of the well was detached by swabbing the well surfaces using a sterile cotton swab. The entire 2 ml content of the well along with the cotton swab was placed into a 15 ml polypropylene tube and diluted with 8 ml of 0.1% peptone water which was vigorously vortexed for 2 min. After vortexing and 100 µl subsamples were serially diluted using a 0.8% NaCl solution. From each diluted sample, 100 µl aliquots were spread plated onto TSA plates and plates were incubated at 37°C for 24 h to determine CFU

3.2.9 Interference at higher concentrations of essential oils in the crystal violet staining assay

This assay was performed to eliminate the possibility of essential oils of thyme, oregano and carvacrol interfering with the staining of biofilm mass used in quantification of biofilms. The essential oils of thyme, oregano and carvacrol were initially diluted in PG (50:50 v/v) and appropriate quantities were added in TSB broth to yield the final essential oil concentrations between 0.012% and 1% in TSB. These essential oil concentrations in TSB were distributed into 24-well microtiter plates (2 ml/well) in triplicate wells and the plates were incubated at 22°C for 24 h. After the incubation, TSB solutions containing essential oils were removed and these plates were washed three times using sterile DI water. Subsequently, the individual wells were stained for 15 min with 1% crystal violet solution and that wells were washed five times with sterile DI water to remove residual crystal violet. The bound crystal violet stain was solubilized with 2 ml of ethyl acetate (80% ethanol and 20% acetone) solution and quantified at OD562nm. This experiment was repeated three times.

3.2.10 Effect of carvacrol on the reduction of *Salmonella* biofilms on stainless steel coupons

The stainless steel coupons (2 × 2 cm; 2B finish) were sterilized by autoclaving at 120 OC and 15 psi for 15 min. Equal volumes of the overnight culture of three *Salmonella* strains (S. Typhimurium ATCC-14028, S. Typhimurium ATCC-19585, and S. enterica ATCC-23564) were first mixed and then serially diluted to achieve an inoculum level of ~ 107 CFU/ml. Hundred microliter of the mixed *Salmonella* cell suspension was spotted on the surface of the stainless steel coupon. After spotting, the coupons were placed in the Petri dish and incubated at 22°C for 24 h to allow biofilm formation. After 24 h incubation, the residual TSB growth medium was removed by

pipetting and the coupons were washed 3 times using sterile water to remove the loosely attached *Salmonella* cells. The washing step included lifting each stainless steel coupon using sterile forceps and gently shaking them in sterile DI water in a Petri dish.

The carvacrol essential oil solution was initially solubilized in propylene glycol and mixed with sterile DI water to obtain the concentrations between 0.012% and 0.1%. The coupons were exposed to 250 µl of the carvacrol solution containing varying concentrations and the surviving cells were recovered after 1 h, 4 h and 24 h by vortexing the coupons with glass beads. Previously, Lindsay and Holy (1997) tested the efficacy of sonication, vortexing, and shaking with beads in recovering L. monocytogenes biofilm cells from the stainless steel coupons and found that they all had similar recovery. At each time point, the essential oil on the surface of the coupons was removed, and the coupons from each treatment were washed three times in sterile DI water to remove residual essential oil content. The washed coupons were placed in a 50 ml tube containing 20 ml of peptone water. The adhering cells were released from the coupons by adding 5 glass beads (5 mm dia) into the tube and vortexing for 2 min. The serial dilutions of these aliquots were made in 0.8% saline and spread plated on TSA. When the recovery of *Salmonella* following the essential oil treatments was expected to be low, 500 µl volumes of each samples was spreaded on four separate plates to increase the detection sensitivity. These plates were incubated at 37°C for 24 h and the results were reported in CFU/ ml.

3.3 Results

3.3.1 Antimicrobial efficacy of nine different essential oils against *Salmonella*

The antimicrobial activity of nine different essential oils against the reference strain of S. Typhimurium ATCC-14028 was dependent on both essential oil and type of media used, i.e. TSA or XLD plates (Table 3.1). Typically, higher zones of inhibition were observed on XLD plates when compared to TSA plates. Essential oils of thyme, oregano and carvacrol had more pronounced antimicrobial activity as determined by the zone of inhibition. These three essential oils yielded between 24 mm and 52 mm zones of inhibition in TSA plate and almost 80 mm zones of inhibition in the XLD plates. Lemon and orange essential oils were least effective since they showed no zones of inhibition on either TSA or XLD plates. Antimicrobial activities of cinnamon, eugenol, bay and linalool essential oils were moderate, yielding 11 to 24 mm inhibition zones in TSA plates and between 26-35 mm in XLD plates (Table 3.1)

3.3.2 Antimicrobial activity of selected essential oils against different strains of *Salmonella* spp.

Table 3.2 shows the antimicrobial efficacy of essential oils of thyme, oregano and carvacrol by disk-diffusion assay against S. Typhimurium ATCC-14028, S. Typhimurium ATCC-19585 and S. enterica ATCC-23564. No appreciable differences in zones of inhibition were observed between three strains of *Salmonella* on TSA plates or XLD plates. However, as seen during the screening of all nine essential oils, higher zones of inhibitions were observed in XLD plates in comparison to TSA plates. In XLD, all three strains yielded near complete 80 mm zones of inhibition. On TSA, thyme oil produced between 36 to 51 mm zones of inhibition against all three *Salmonella* strains. Carvacrol produced between 24 to 35 mm zones of inhibition for these three *Salmonella*

strains. Some strain specific variations were observed against oregano oil in TSA plates. The strain S. enterica ATCC 23564 produced a relatively larger zone of inhibition of 69 mm compared to other two strains which showed between 26 to 34 mm zones of inhibition in TSA plates.

3.3.3 Temperature and *Salmonella* strain dependent variation in biofilm formation

As shown in Figure 3.1, all three *Salmonella* strains produced biofilms in 24-well microtiter assays exhibiting significant differences in biofilm formation in terms of strain variations and temperature at which biofilms were produced. The biofilm production as determined by OD562nm readings, were higher at 22°C when compared to 30°C or 37°C in S. enterica and S. Typhimurium ATCC 19585 strains. For S. Typhimurium ATCC 19585, OD562nm readings was 1.2 at 22°C, 0.8 at 30°C and 0.5 at 37°C with biofilm levels at all these three temperature being different from each other ($P \leq 0.05$). For S. enterica and S. Typhimurium ATCC 14028, the biofilm formations were not ($p > 0.05$) between 22°C and 30°C but these were significantly different compared to the biofilm produced at 37°C. Between the three strains, S. enterica ATCC 23564 and S. Typhimurium ATCC 19585 produced more pronounced biofilms at 22°C and 30°C compared to that produced by S. Typhimurium ATCC 14028.

3.3.4 MIC and MBC measurements of selected essential oils against *Salmonella* spp.

There was no growth as measured by turbidity at OD630nm after 24 h at 22°C for all three *Salmonella* strains in the presence of thyme and oregano and carvacrol essential oils at 0.025%. Hence, the MIC values for all three *Salmonella* strains against thyme, oregano and carvacrol were 0.025%. Moreover, no CFU counts were recovered when

these aliquots from 0.025% treatment where spread plated on TSA plates. Hence, the MBC values were also 0.025% for all three *Salmonella* strains

3.3.5 Interference to absorbance measurements at 562 nm in crystal violet assay by higher concentrations of essential oils

Figure 3.2 shows the interaction of essential oils of thyme oregano and carvacrol at varying concentrations with crystal violet stain used in biofilm quantification. This assay was performed to ensure that the selected essential oil concentrations did not interfere with the crystal violet staining protocol used in biofilm inhibition and eradication assays. None of the essential oils when used at concentration of 0.2% or lower showed any interference with crystal violet stain as it was evident by identical OD readings as compared to the control treatments (no addition of essential oils). For thyme and oregano, 0.4% or higher essential oils concentrations interfered with the crystal violet staining and generated the false positive reaction which corresponded to the drastic increase in the OD562nm readings. The carvacrol concentration up to 0.5% had no effect on the crystal violet staining assay but 1% concentrations resulted in false (higher) OD562 measurements.

3.3.6 Effect of sublethal concentrations of essential oils on *Salmonella* spp. biofilm formation

Figure 3.3 shows the antimicrobial efficacy of essential oils of thyme and oregano and carvacrol at 0.006% (1/4 MIC) and 0.012% (1/2 MIC) that can inhibit *Salmonella* spp. biofilm formation in 24-well microtiter plates at 22°C. Essential oils of thyme, oregano and carvacrol were tested at 0.006% (1/4 MIC) and 0.012% (1/2 MIC) concentrations. Prior to this experimentation, we checked to make sure that there were no differences in growth turbidity (OD630nm) following 24 h incubation at 22°C in the

presence of thyme, oregano and carvacrol at 0% (control), 0.006% and 0.012% (data not shown here).

Compared to the control, all three essential oils at 0.012% reduced ($p< 0.05$) the amount of biofilm produced by the three *Salmonella* strains. At 0.006% of thyme and oregano, S. Typhimurium ATCC 19585 and S. enterica ATCC 23564 biofilm were not reduced ($p>0.05$); it was reduced for these two strains at 0.006% carvacrol. For S. Typhimurium strain ATCC 14028, all three essential oils at 0.006% concentration were able to reduce ($p<0.05$) the biofilm production. The degree of biofilm inhibition also varied with the biofilm mass produced in the control samples that received no essential oil treatment. In general, a higher concentration of biofilm was observed following the essential oil treatment of S. Typhimurium ATCC 19585 and S. enterica ATCC 23564 in comparison to the S. Typhimurium strain ATCC 14028, which was partially due to the fact that the aforementioned two strains also resulted in relatively higher biofilm production in control samples.

3.3.7 Effect of sublethal and lethal concentrations of essential oils on the reduction of preformed biofilms of *Salmonella* spp.

Figure 3.4 shows the antimicrobial efficacy of the essential oils of thyme and oregano and carvacrol at 0.012%, 0025% 0.05% and 0.1% at eradicating 1-day-old *Salmonella* spp. biofilms in 24-well microtiter plates at 22°C. This experiment was performed to evaluate if the essential oils of thyme and oregano and carvacrol process the ability to reduce preformed biofilms by *Salmonella* strains. Compared to control samples, no differences were detected ($p>0.05$) in any *Salmonella* strains in terms of biofilm reduction following essential oil treatments at 0.012% which is the 1/2 MIC concentration. Also at 0.025% of thyme and oregano, which is also the MIC and MBC

for the planktonic cells, there was no significant reduction in the preformed biofilms for any *Salmonella* strains except for the reduction of S. Typhimurium 14028 ($p<0.05$) when 0.025% oregano oil was used. However, 0.025% carvacrol reduced ($p<0.05$) the amount of preformed biofilms for all three strains. Treatment of 24 h old biofilms with thyme, oregano or carvacrol at 0.05% or 0.1% significantly reduced the amount of preformed biofilms. The OD562nm reading for control wells were between 1.0 to 1.5 for all three *Salmonella* strains whereas treatments with 0.05% or 0.1% reduced the biofilm levels down to ~0.3 units. Also, the OD562nm measurement did not differentiate the biofilm reductions between 0.05% and 0.1% essential oil treatments.

3.3.8 Time dependent reduction in preformed *Salmonella* biofilm by essential oils

The amount of mixed *Salmonella* spp. biofilm cells that were recovered after 1, 4 and 24 h incubation in control samples (treatment with sterile DI water) were between 6.8 to 7.4 log CFU/well. Treatment with 0.025% thyme reduced the *Salmonella* biofilm population by about 1 log CFU/well within 1 h and by 1.3 log CFU/well after 4 h. However, there was no further meaningful decrease in biofilm mass when the treatment exposure was prolonged to 24 h. With both oregano and carvacrol, there was a proportionally higher inactivation of *Salmonella* biofilm cells with an increase in the treatment exposure time. With 0.025% oregano treatment, these reductions were 0.7, 1.9 and 3.6 log CFU/well following 1, 4 and 24 h time period, For 0.025% carvacrol, these reductions were 1.6, 3.4 and 4.9 log CFU/well within 1, 4 and 24 h exposure time, respectively. Treatment of preformed biofilms with these three essential oils at 0.05% and 1% reduced the biofilm cells to undetectable level within 1 h (1 log CFU/well minimum

detection sensitivity). These biofilm reductions were maintained at both 4 and 24 h exposure intervals indicating that there was no regrowth of the biofilm cell mass.

3.3.9 Efficacy of carvacrol in reducing mixed strain biofilms of *Salmonella* spp. on stainless steel coupons

The amount of mixed *Salmonella* biofilm cells recovered from a 2×2 cm coupon surface was ~7 log CFU/coupon. Treatment of these stainless steel coupons containing *Salmonella* biofilm cells with 0.012% of carvacrol did not result in any reduction in biofilm cell counts. Also, treatment with 0.025% of carvacrol did not show any reduction in biofilm counts within 1 h while there was 2.2 log CFU/coupon reduction within 4 h exposure and a more pronounced 3.9 log CFU/coupon reduction with 24 h exposure time. Treatment with 0.05% and 0.1% carvacrol were highly effective at inactivating these biofilm cells mass by decreasing the *Salmonella* biofilm population to an undetectable level within 1 h.

3.4 Discussion

In our assay, we initially tested the biofilm formation at three temperature regimes which were 22°C, 30°C and 37°C. The optimal growth of *Salmonella* occurred between 30°C and 37°C, however, these optimum higher temperatures are not very relevant to the food processing environments. Nevertheless, screening of biofilm mass at these three temperatures suggested that the optimal temperature for *Salmonella* biofilm formation for three strains was 22°C which is the normal room temperature (Figure 3.1). Prior studies also show that the optimal biofilm formation in *Salmonella* occurs at around 20°C (Giaouris and others 2005; Stepanovic and others 2003). Previously, Knowles and others (2005) reported that a mixed species biofilm that included S. Typhimurium matures and

reaches the quasi-steady state at which microenvironments inside these biofilm matrices become stable enough not to be affected by the external selective pressure or nutrient depletion and as a result of such microstructures, it ultimately shows resistance to antimicrobial perturbation. In the assay, biofilm mass was allowed to form on the polystyrene 24-well microtiter well plate surfaces or stainless steel coupon surfaces for 24 h. The biofilm mass was expected to reach a steady-state during this time period. The integrity of the biofilm mass was evident from the fact that after the biofilm formation, when the microtiter wells or coupons were vigorously rinsed 3 times, it still released about 7-8 log CFU of mixed *Salmonella* cells from the attached polystyrene well surfaces or stainless steel coupons.

In these experiments, we evaluated the ability of selected essential oils to inhibit the biofilm formation and also to eradicate preformed biofilms using a crystal violet staining procedure. The crystal violet based assay is routinely used to quantify biofilm formation for the following reasons: (a) to measure the strain variation (Borucki and others 2003; Li and others 2003); (b) to measure the effect of selective mutation in targeted genes (Sandberg and others 2008); (c) to observe environmental manipulation (Giaouris and others 2005); and (d) also to evaluate the efficacy of antimicrobial substances (Polonio and others 2001; Richards and others 2008). Since crystal violet staining does not differentiate between live and dead organisms, any quantification of the biofilm cell mass following antimicrobial treatment actually depends on the mode of action of these antimicrobial substances. We employed essential oils of thyme, oregano and carvacrol as natural biocides. Previously, it has been shown that carvacrol at non-biocidal concentrations disrupts the normal biofilm formation by arresting the cell mass at the microcolony stage (Knowles and others 2005). Nostro and others (2007) suggested

an alternative hypothesis that these essential oils at non-lethal concentrations could interact with surface proteins of the bacterial cells and thereby reduce the bacterial cell attachment to the target surface. Another possible mechanism could be the reduced motility and flagella production in the presence of these essential oils as noted previously for carvacrol at a non-biocidal concentrations which reduced flagella synthesis and motility in the E. coli O157:H7 cells (Burt and others 2007). Elsewhere, inhibition of bacterial biofilm formation was also observed on E. coli K-12 cells by a ground beef derived small chain fatty acid (Soni and others 2008) and by citrus flavonoids and limonoids on E. coli 157:H7 and V. harveyi cells (Vikram and others 2010; Vikram and others 2011).

At lethal concentrations, it has been proposed that the essential oil biocides interact with lipid bi-layer of cytoplasmic membranes which results in the damage and loss of integrity of the cell membrane with a subsequent leakage of the cellular material from inside the cells (Ultee and others 1999; Burt, 2004). Moreover, active essential oil components of thymol and carvacrol due to their hydrophilic properties and a strong antimicrobial activity are theorized to penetrate through the polysaccharide membrane of the biofilm matrices and such diffusion causes the detachment of the biofilm cell mass (Nostro and others 2007). Nevertheless, the quantitative data derived from the crystal violet based quantification of the biofilm mass in our experiment suggests that the essential oils of thyme and oregano and carvacrol are able to both inhibit and eradicate biofilm formation in a concentration dependent manner against *Salmonella* spp. Such reductions in biofilm mass by essential oil treatments using crystal violet staining based quantification has been previously shown for the S. aureus and S. epidermidis (Nostro

and others 2007), L. monocytogenes and E. coli O157:H7 (Perez-Conesa and others 2006).

Biofilm mass reductions that were observed in this study with OD562nm readings were also confirmed using CFU counts. There was a high degree of correlations in biofilm reductions observed by these two approaches, i.e. proportional reduction in crystal violet stained biofilm cell mass at a particular essential oil treatment also matched with a proportional decrease in the biofilm CFU counts. This high level of correlation between OD562nm and CFU counts suggest that the crystal violet based rapid quantification is a suitable rapid alternative method to evaluate the efficacy of essential oils or against any other antimicrobial compounds capable of detaching the cells of biofilm mass.

Wong and others (2000) previously studied various disinfectants and the age of the biofilm age as determinant of sanitation efficacy. The 7-day-old biofilm did not have any measurable higher resistance when compared to the 3- or 5-day-old biofilms when sodium hypochlorite, ethanol or quaternary ammonium compounds were used (Wong and others 2000). However, the increase in the contact exposure time or concentrations of disinfectant showed proportionally higher sanitation efficacy. In our assay, we did not evaluate the effect of tested essential oils with the age of biofilm as a variable. However, our assay with 1-day-old biofilm also indicated both concentrations and time dependent inactivation of *Salmonella* biofilm mass (Table 3.3 and Table 3.4). In particular, the treatment of biofilm cells at 0.025% of the treatment produced a corresponding time dependent reduction with an increased exposure time leading to a higher reduction of biofilm counts. At 0.05% and 0.1%, the effect was much more pronounced and therefore no effect of exposure time was observed since these essential oil concentrations

completely eliminated biofilm counts in both 24-well microtiter assay and stainless steel coupons within 1 h.

Resistance and subsequent adaptation of biofilm cells to any antimicrobials by a frequent repetitive exposure is a great concern. Several studies have reported that the biofilm cells in general are more resistant compared to their planktonic counterparts (Spoering and Lewis, 2001; Anderson and O'Toole, 2008; Jacques and others). In this assay, 0.025% concentrations of all three essential oils were sufficient to kill 7 log CFU/ml of *Salmonella* cells in the planktonic culture whereas slightly higher 0.05%-1% essential oil concentrations were needed to inactivate the 7-8 log CFU in the 1-day-old biofilm mass. In terms of the adaptive responses, it was previously reported that the exposure of *Salmonella* Enteritidis biofilms to the sub-lethal concentrations of benzalkonium chloride for 7 days resulted in an adaptive response in biofilm cells which have shown resistance even up to a 500-fold increase in benzalkonium chloride concentrations (Mangalappalli-Illathu and Korber, 2006). With respect to essential oils, it has been proposed that since each essential oil is made up of several antimicrobial constituents, there is less chance of adaptive resistance and the larger antimicrobial constituents that are present in a single essential oil can serve as a hurdle response to bacterial cell adaptation (Daferera and others 2003).

3.5 Conclusions

In conclusion, the data presented in this work shows that the essential oils of thyme, oregano and carvacrol show strong antimicrobial activity against the *Salmonella* biofilm cells that are present on polystyrene or stainless steel coupon surfaces. The biofilm matrices produced in food processing plant environments will be multispecies

and also influenced by the other processing environmental related parameters such as presence of food residue or sublethal exposure to various stresses. Hence, further experiments are needed to evaluate the effect of such parameters on the efficacy of essential oils at reducing the mass of *Salmonella* biofilm cells in the simulated food processing environment.

Table 3.1 Screening of nine essential oils against *S.* Typhimurium (ATCC 14028) through use of a disk-diffusion assay on non-selective and selective agars

Essential oils	Inhibition zone (mm)	
	TSA	XLD
Bay oil	16 ± 2	44 ± 2
Carvacrol	24 ± 1	80 ± 0
Cinnamon leaf oil	10 ± 2	26 ± 2
Eugenol	11 ± 1	20 ± 4
Lemon oil	0	0
Linalool	0	45 ± 3
Orange oil	0	0
Oregano oil	34 ± 1	80 ± 0
Thyme oil	52 ± 2	80 ± 0
Propylene glycol	0	0

Note: TSA = Tryptic soy agar. XLD = Xylose lysine deoxycholate agar

Table 3.2 Screening of thyme, oregano and carvacrol essential oils against three strains of *Salmonella* spp. through use of a disk-diffusion assay on non-selective and selective agars

Salmonella spp.	Inhibition zone (mm)					
	Thyme		Oregano		Carvacrol	
	TSA	XLD	TSA	XLD	TSA	XLD
S. enterica ATCC 23564	51 ± 4	80 ± 0	69 ± 3	80 ± 0	24 ± 1	80 ± 0
S. Typhimurium ATCC 19585	36 ± 2	80 ± 0	26 ± 5	80 ± 0	26 ± 3	71 ± 3
S. Typhimurium ATCC 14028	46 ± 1	80 ± 0	34 ± 3	75 ± 4	35 ± 4	70 ± 1

Note: TSA = Tryptic soy agar. XLD = Xylose lysine deoxycholate agar

Table 3.3 Effect of essential oil concentration and contact time on the reduction of three-strain mixed *Salmonella* biofilm in polystyrene 24-well microtitter plates

Essential oil concentrations	*Salmonella* spp. (Log CFU/well)								
	Thyme			Oregano			Carvacrol		
	1 h	4 h	24 h	1 h	4 h	24 h	1 h	4 h	24 h
0 %	7.1±0.3	6.8±0.7	7.4±0.1	7.1±0.3	6.8±0.7	7.4 ± 0.02	7.1 ± 0.3	6.8±0.7	7.4±0.1
0.025%	6.1±0.1	5.5±0.2	5.2±0.2	6.4±0.3	4.9±0.1	3.8±0.1	5.5 ±0.02	3.4±0.1	2.5±0.5
0.05%	ND	ND	ND	ND	ND	ND	ND	ND	ND
0.1%	ND	ND	ND	ND	ND	ND	ND	ND	ND

Note: ND = Not detected. Minimum detection limit is 1 log CFU/well.

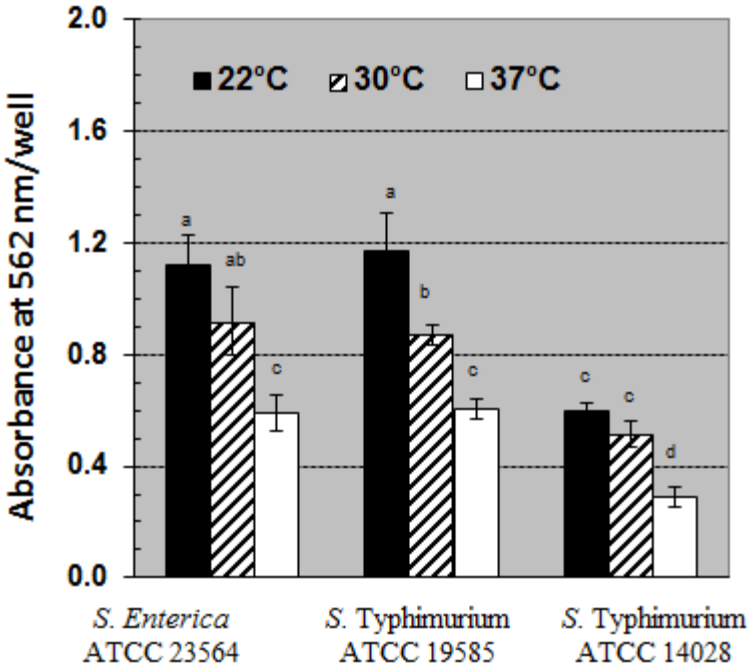

Figure 3.1 Effect of temperature on the biofilm formation by *Salmonella* spp. in polystyrene 24-well microtiter plates.

Note: Bars with different lowercase letters indicate differences in mean for *Salmonella* reduction through use of different essential oils at varying concentrations based on one-way ANOVA posthoc tukey test ($P \leq 0.05$)

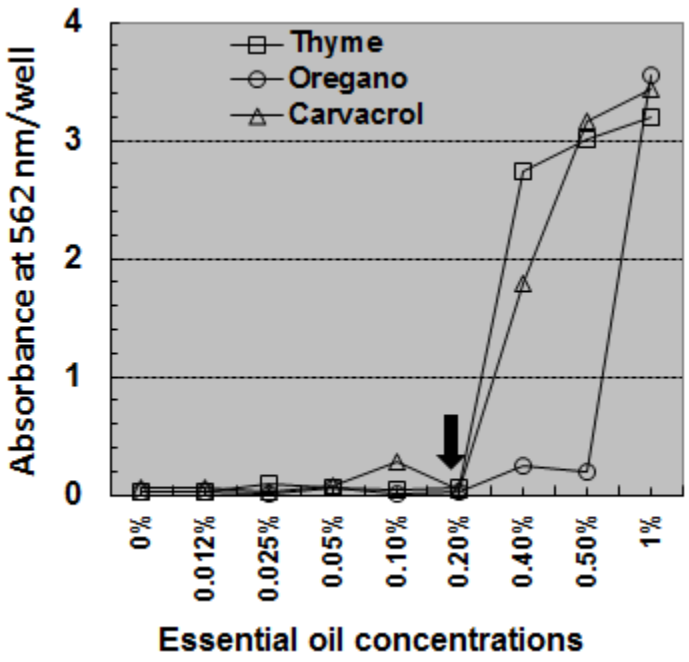

Figure 3.2 Interference in the absorbance measurements at in the crystal violet assay when essential oil concentrations greater than 0.2% were used (562 nm).

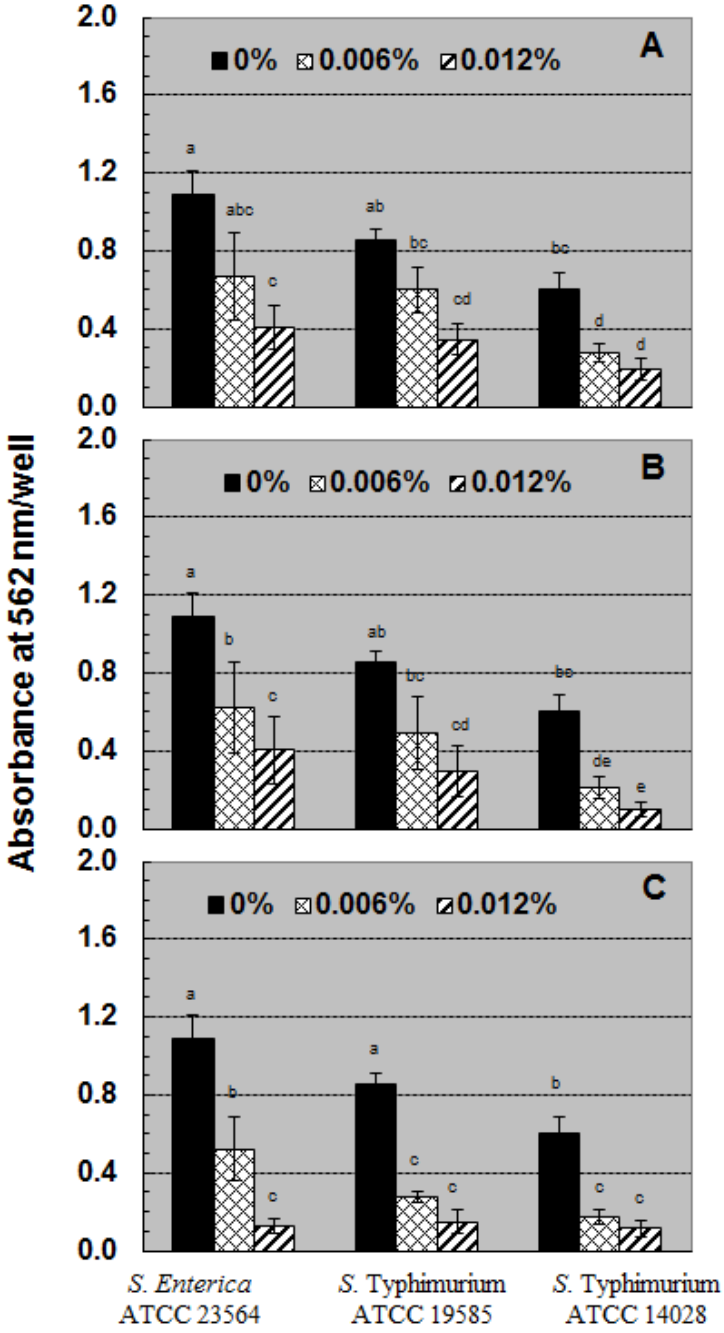

Figure 3.3 Effect of sublethal concentrations of essential oils of: (A) thyme, (B) oregano, and (C) carvacrol on *Salmonella* spp. biofilm formation in polystyrene 24-well microtiter plates at 22°C.

Note: Bars with different lowercase letters indicate differences in mean for *Salmonella* reduction by different essential oils at varying concentrations. Mean separation was conducted using tukey test p specified α- level = 0.05based on one-way ANOVA posthoc tukey test ($P \leq 0.05$).

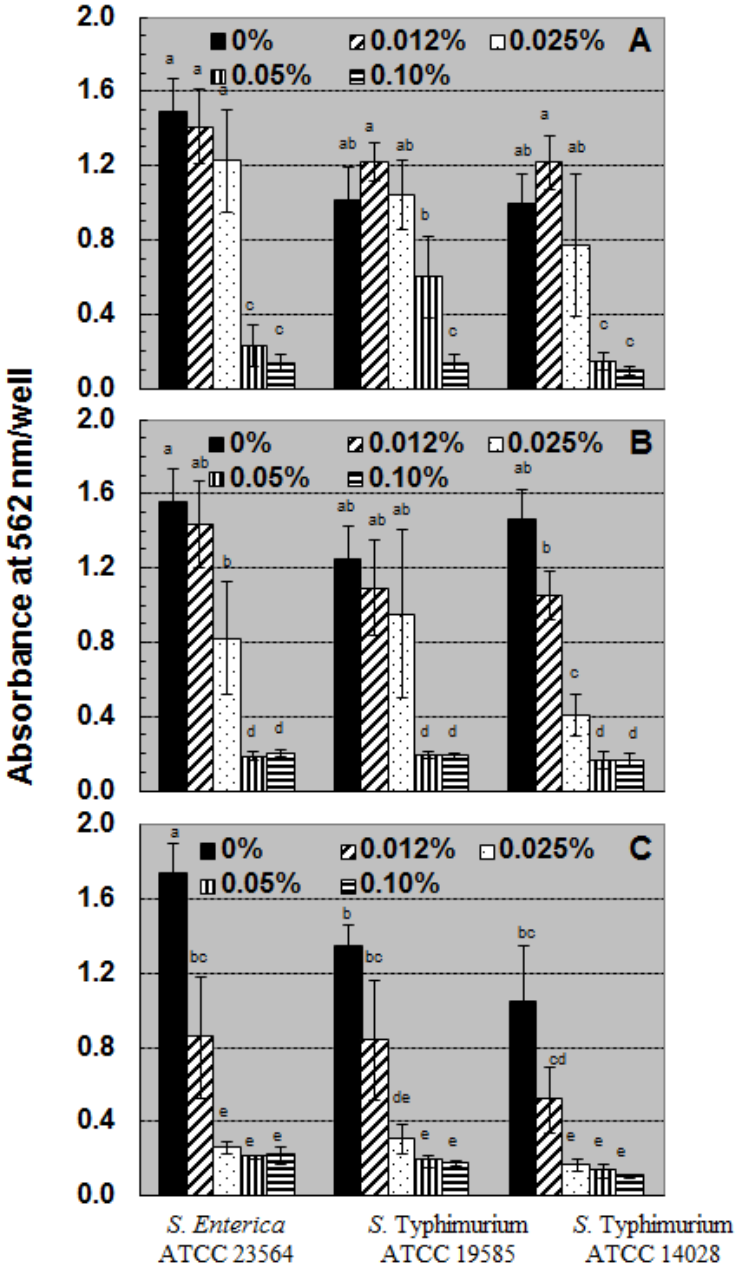

Figure 3.4 Effect of sublethal and lethal concentrations essential oils of: (A) thyme, (B) oregano, and (C) carvacrol on reduction of *Salmonella* spp. biofilms in 24-well microtiter plates at 22°C.

Note: Bars with different lowercase letters indicate differences in mean for *Salmonella* reduction by different essential oils at varying concentrations. Mean separation was conducted using tukey test p specified α- level = 0.05 based on one-way ANOVA posthoc tukey test ($P \leq 0.05$).

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CHAPTER IV

SYNERGISTIC ACTIVITY BETWEEN LAURIC ARGINATE AND CARVACROL IN REDUCING *SALMONELLA* SPECIES IN GROUND TURKEY CONTAINING 1%, 7% AND 15% FAT

4.1 Introduction

Salmonella is an important foodborne pathogen that causes Salmonellosis in infected individuals. Poultry breeding and processing has been associated with *Salmonella* and consequently this pathogen has been frequently associated with various retail poultry products including chicken and turkey carcasses and ground turkey meat (McPherson and others 2006; Snow and others 2011; Aury and others 2010; Reynolds and others 2010). FoodNet-CDC in the year 2009 reported 7039 laboratory confirmed cases of *Salmonella*. Further serotyping of laboratory collected isolates suggested that the three serotypes, Enteritidis, Typhimurium, and Newport were the most prevalent strain and accounted for 19.2%, 16.1% and 12.1% prevalence, respectively (MMWR, 2010). Among S. Typhimurium strains antimicrobial resistance is quite common for up to five antibiotics (Glenn and others 2011; Graziani and others 2008). Moreover, *Salmonella* serotype Heidelberg is also gaining attention due to its multidrug resistance and its recent increased association with foodborne outbreaks (Han and others 2011; Berrang and others 2009). Zhao and others (2008) analyzed ~10,000 retail samples of ground turkey and chicken and identified 228 Heidelberg from these samples. Of these, 49 isolates were resistant to five antimicrobials and eight were resistant to nine antimicrobials. Most

recently in 2011, a S. Heidelberg strain that were resistant to a number of commonly used antibiotics was associated with the consumption of ground turkey which infected 77 people in 26 states in the United States (CDC, 2011).

Since poultry is a potential source for the dissemination of *Salmonella* spp. in the food chain, significant efforts have been directed towards identifying the control measures at both pre-harvest production and post-harvest processing stages. Much of these measures have been standardized by better management practices including, effective sanitation of the breeding facility and continuous monitoring of *Salmonella* prevalence, proper handling and storage, and incorporation of HACCP (Vadhanasin and others, 2004). Also, the use of probiotic cultures during the feeding of chicks and turkey poults resulted in 2 log CFU/g reductions in of S. Heidelberg counts when compared to the control samples that contained 3 log CFU/g (Menconi and others 2011). Similarly, other studies also demonstrated the effectiveness of different probiotic organisms for *Salmonella* reductions in broilers (Higgins and others 2007; Vicente and others 2008). Incorporation of a small chain fatty acid such as butyric acid sodium salt has also shown effectiveness in reducing *S.* Enteritidis in broiler chicken (Fernandez-Rubio and others 2009). Nevertheless continuous association of *Salmonella* in retail poultry products suggest that even if pre-harvest strategies can aid in reducing *Salmonella* persistence, additional control measures against this pathogen at the postharvest processing steps are very important (Bucher and others 2011).

A variety of antimicrobial treatments such as acid wash (Laury and others 2009), acidic calcium sulfate (Benli and others 2011), and electrolyzed oxidizing water (Fabrizio and others 2002) have been evaluated for poultry products. However, all these antimicrobial processes are not acceptable in poultry production due to quality challenges

(Cagri-Mehmetoglu, 2011). Use of organic acid wash at an elevated concentration causes yellowness in the carcasses (Bilgili and others 1998). Irradiation treatment, while effective in reducing microbial load from chicken breast, caused lower texture and flavor attributes following 14 day refrigerated storage (Lewis and others 2002). In other studies, use of a four strain mixture of *Lactobacillus* at 6 log CFU/g on ground turkey meat reduced a three strain *Salmonella* mixture of Typhimurium, Enteritidis and Heidelberg by 2 log CFU/g when compared to a5 log CFU/g *Salmonella* mixture detected in the control samples after 24 h refrigerated storage (Dow and others 2011).

Essential oils and recently FDA-approved lauric arginate are some of the important antimicrobial agents that can be used to control pathogenic microorganisms in food products. Essential oils are aromatic liquids derived from plant extracts through steam distillation. Essential oils, in particular thyme, oregano and their active phenolic constituent carvacrol, are known for their broad-spectrum activity against both Gram-positive and Gram-negative microorganisms (Hammer and others 1999; Dorman and Deans, 2000; Cutter, 2000; Delaquis and others 2002). However, there has been a very limited amount of work on optimizing the use of essential oils in food products (Burt, 2004). Lauric arginate (LAE) (ethyl N Lauroyl L-arginate Hydrochloride) is an FDA approved broad spectrum food preservative at concentrations up to 200 ppm (USFDA, 2005). The antimicrobial activity of LAE is based on the disruption/instability of the plasma membrane lipid bilayer over a wide pH range (3-7). The present study evaluated the efficacy of carvacrol and LAE against a three strain mixture of *Salmonella* spp. in ground turkey with the following objectives: a) to determine the temperature dependent efficacy of carvacrol and LAE against a three strain mixture of *Salmonella* spp.; b) to evaluate the antimicrobial efficacy of carvacrol and LAE at varying concentrations in

ground turkey samples containing 15%, 7% and 1% fat against a mixed *Salmonella* spp.; and c) to determine the synergistic action of carvacrol and LAE against three strain mixture of *Salmonella* spp. in ground turkey.

4.2 Material and Methods

4.2.1 *Salmonella* strains

Three *Salmonella* strains; *S.* Typhimurium ATCC 14028, *S.* Typhimurium ATCC 19585, and *S. enterica* ATCC 23564, were used in this study. These strains were maintained in tryptic soy broth (TSB) slants at 4°C and a working culture of these strains were prepared by inoculating 10 µl of stock into 10 ml TSB and incubation at 37°C for 18 h to obtain a cell concentration of ~10^9 CFU/ml. To obtain a mixed cell suspension of these *Salmonella* strains, 1 ml of cell suspensions of each of three strains that were grown overnight were mixed into 7 ml of TSB. The mixture was serially diluted in 0.8% saline to achieve the desired cell concentrations.

4.2.2 Antimicrobial agents

Carvacrol essential oil was purchased from Sigma Aldrich (≥ 98% purity; Sigma Aldrich, St. Louis, MO, United States). For the experimental purpose, the carvacrol solution was solubilized by mixing a 50% essential oil with 50% propylene glycol (PG). Mirenat-TT lauric arginate (LAE) was obtained from Vedeqsa (Vedeqsa Inc., New York, NY 10001). The Mirenat-TT solution is commercially available and contains 10% active LAE that is dissolved in propylene glycol (solvent) and polysorbate 20 (emulsifier).

4.2.3 Effect of temperature on the antimicrobial efficacy of carvacrol and lauric arginate against three strain mixture of *Salmonella* spp. in tryptic soy broth

In this assay, efficacy of carvacrol and LAE at different temperatures was assessed using a three strain mixture of *Salmonella* spp. The concentrations ranging from 0.025 to 0.2% for carvacrol and 25 ppm to 200 ppm for LAE and were prepared in TSB as follows: carvacrol was serially 2-fold (50:50 v/v) in PG and 100 µl from each dilution was added into 25 ml of TSB to achieve the desired carvacrol concentrations of 0.2%, 0.1%, 0.05%, and 0.025%. For LAE treatment, 50 µl of 10% LAE solution was added into 25 ml of TSB to yield a 200 ppm LAE concentration, and the 200 ppm LAE solution was 2-fold serially diluted in TSB to yield LAE concentrations of 200 ppm, 100 ppm, 50 ppm and 25 ppm. The control treatment did not receive any antimicrobial. One-ml volumes of these TSB solutions containing different concentrations of antimicrobials were distributed into the eppendorf tubes. Subsequently, the eppendorf tubes containing these antimicrobial concentrations were placed at 4°C, 22°C (room temperature) and 45°C. For incubation at 45°C, a heating block was used to maintain a constant temperature. Duplicate tubes were used for each antimicrobial concentration and temperature treatment. Tubes were incubated at the treatment temperature for 2 h to achieve temperature equilibration. Following this step, 10 µl of 108 CFU/ml of *Salmonella* mixed culture was added into each tube. During the inoculation step, the 4°C sample tubes were placed on ice to minimize temperature abuse. For 45°C samples, the addition of inoculum was performed at room temperature but the samples were immediately placed at 45°C following inoculation. The *Salmonella* inoculated samples were either incubated at a respective temperature for 30 min and then 100 µl aliquots were spread plated directly or after 10-fold serial dilutions into TSA. These plates were incubated at 37°C for 24 h and colony forming units (CFU) were recorded.

4.2.4 Synergistic action between lauric arginate and carvacrol on three strain mixture of *Salmonella* spp. in tryptic soy broth

Various concentrations of carvacrol and LAE either individually or in combinations were tested in TSB at 4°C and 22°C to evaluate, if there exist, any synergistic activity between these two antimicrobials. This assay was performed in 96-well microtiter plates. Initially, LAE solutions in TSB at 400 ppm, 200 ppm, 100 ppm, 50 ppm, 25 ppm, 12 ppm, 6 ppm and 0 ppm and carvacrol solutions in TSB at 0.4%, 0.2%, 0.1%, 0.05%, 0.025%, 0.012%, 0.006% and 0% were prepared as described earlier in section 4.2.3.

In a 96-well microtiter well plate, LAE solutions at different concentrations were added vertically and carvacrol concentrations at different concentrations were added horizontally. Each LAE concentration solution at a 100 µl/well was added into eight wells of one vertical row and eight vertical rows were used for eight different LAE concentrations. In a similar way, the eight wells of each horizontal row were filled with 100 µl solution of different carvacrol concentrations. This resulted in an 8×8 matrix for each eight LAE and carvacrol concentrations in combinations. For 4°C treatments, the plates were incubated for2 h in a refrigerated incubator for temperature equilibration prior to inoculation step with a three stain mixture of *Salmonella*. To maintain experimental consistency, 22°C plates were also kept at a room temperature for 2 h prior to inoculation. For inoculation, each well was added with 20 µl of 107 CFU/ml *Salmonella* mixed strain cell suspension was added to each well. During the inoculation step, the 4°C microtiter plate was kept on ice to prevent temperature abuse. Microtiter plates were briefly placed in a shaking incubator for 30 sec for mixing of the inoculum within the wells. These plates were incubated at 4°C or 22°C for 30 min prior to performing *Salmonella* enumeration.

As a preliminary screening, 25 µl aliquot from each well was directly spotted on TSA agar plates and incubated for 24 h at 37°C. This provided qualitative measurements for the presence or absence of *Salmonella*. Based on this information, in the next round of experimentation, a 100 µl aliquot from each of the 12 to 15 wells from each plate (from total 48 filled wells) were spread plated either directly or after their serial dilution in 0.8% NaCl saline on TSA to obtain the quantitative *Salmonella* CFU counts.

4.2.5 Effect of lauric arginate and carvacrol against three strain mixture of *Salmonella* spp. and total microbial load in ground turkey

Samples of ground turkey containing 1% (24.21% total solid), 7% (26.1% total solid) and 15% (31.4% total solid) fat content were purchased from a retail grocery store and samples were brought to the laboratory in an ice-cooler to keep the refrigerated condition at which they were purchased from the store. The samples were aseptically weighed into 10 g subsamples in a BSL-2 cabinet and then samples were placed directly into vacuum bags. On the surface of each sample, 100 µl of the 107 CFU/ml *Salmonella* mixed cell suspension was added and this inoculum was thoroughly mixed into ground turkey samples for 1 min by hand massaging the vacuum packages by pressing from the outside. These *Salmonella* challenged ground turkey samples were subsequently treated per g basis with 200 ppm, 1000 ppm, 2000 ppm and 5000 ppm of LAE or 0.1%, 0.5%, 1%, 2% and 5% of carvacrol. These antimicrobials were delivered into ground turkey by mixing with 1 ml of sterile DI water. The control treatments included *Salmonella* challenged or non-challenged samples that received 1 ml of sterile DI water instead of the antimicrobial solution. The desired antimicrobial quantity needed for each of the 11 g subsamples (accounting for 10 g ground turkey, 1 ml of antimicrobial volume) were initially prepared in 1 ml of sterile water and was added to the surface of the ground

turkey sample. Carvacrol was initially solubilized (50:50 v/v) in propylene glycol prior to addition in sterile DI water. Following the addition of the antimicrobial solution, the samples were thoroughly mixed again by 1 min of hand massaging. These *Salmonella* challenged and antimicrobial treated samples were vacuum sealed and stored at 4°C for 24 h prior to enumeration of *Salmonella*. For enumeration, each sample bag was aseptically opened and 90 ml of peptone water (0.1% peptone containing 0.02% Tween-80) was added. Samples were homogenized for 2 min in a stomacher (Seward, Model 400C) at 230 rpm. From each sample, 250 µl of stomached homogenate was spread plated on XLD agar either directly or after their serial dilution in 0.8% NaCl. Also when the *Salmonella* recovery was expected to be low after the antimicrobial exposure, 4 x 250 µl aliquots (total 1 ml plating volume) was spread plated on four XLD plates to increase the minimum detection limit. These plates were incubated at 37°C for 48 h prior to performing CFU counts.

Additional samples were also prepared to determine the effect of antimicrobial concentration on total microbial load. For this purpose, all protocols were similar to that described above except that the ground turkey samples were not *Salmonella* challenged. The enumeration of total microbial load was performed using Plate Count Agar (PCA) where the plates were incubated at room temperature (22°C) for 72 h prior to performing CFU counts.

4.2.6 Combined effect of lauric arginate and carvacrol in reducing three strain mixture of *Salmonella* spp. in ground turkey

This assay was designed to evaluate if synergistic relationship existed between LAE and carvacrol in ground turkey. These experiments were performed using 7% fat ground turkey samples challenged with a three strain mixture of *Salmonella* inoculum.

The antimicrobial combination treatments used per g of ground turkey meat were: a) mixture of 1% carvacrol and 200 ppm LAE; and b) mixture of 1 % carvacrol and 2000 ppm LAE. Each antimicrobial mixture was delivered in 1 ml sterile water to 10 g of ground turkey. Initially, 2X concentration of each antimicrobial was prepared in water and 500µl of each solution was mixed resulting in the addition of 1 ml of mixed antimicrobial solution was added onto 10 g turkey sample placed in the vacuum packaging bags as described previously. The control treatment samples only received 1 ml of sterile water. Ground turkey samples that received antimicrobials or DI water (control) were stored at 4°C/24 h prior to performing *Salmonella* spp. enumeration as described earlier.

4.2.7 Statistical design

All experiments were repeated three times with two replicates. *Salmonella* spp and total microbial load data were initially converted and expressed as CFU/ml of CFU/g using Excel spread sheet. When desired, the mean significant differences across different treatments were calculated using ANOVA tukey posthoc test using SPPS statistical analyses software package (SPSS version 12.0, Chicago, IL).

4.3 Results

4.3.1 Temperature dependent reduction of *Salmonella* treated with lauric arginate and carvacrol in tryptic soy broth

Incubation temperature had a pronounced effect on the concentrations of LAE and carvacrol that were required to inactivate a three strain mixture of *Salmonella* in TSB. Proportionally higher reductions in *Salmonella* were attained at a higher incubation temperature in TSB (Table 4.1). At 4°C, 200 ppm of LAE exposure for 30 min was

required to inactivate ~6 log CFU/ml of three strain mixture of *Salmonella*. While at 100 ppm of LAE, there were only ~3.6 log reductions in *Salmonella* spp. LAE concentrations of 50 ppm or lower failed to reduce the three strain mixture of *Salmonella* at 4°C in TSB. Conversely, 50 ppm LAE treatment at 22°C or 45°C was sufficient to decrease the mixed *Salmonella* spp inoculum to undetectable levels from the initial inoculum level of ~6 log CFU/ml. The concentration of carvacrol that was required to inactivate 6 log CFU/ml of mixed *Salmonella* to an undetectable level in TSB following a 30 min exposure time were 0.1%, 0.05% and 0.05% at 4°C, 22°C and 45°C, respectively. At 0.025%, there was no significant reduction in *Salmonella* count at 22°C while the counts were reduced by marginal 1.4 log CFU/ml at 45°C (Table 4.1).

4.3.2 Synergistic activity between lauric arginate and carvacrol in tryptic soy broth

There was a synergistic biocidal activity observed at both 4°C and 22°C when sub lethal concentrations of LAE or carvacrol were mixed with each other (Table 4.2). At 4°C, 25 ppm or 50 ppm LAE and 0.025% of carvacrol when applied individually did not result in any appreciable reductions in the initial 5.6 log CFU/ml of the three strain mixture of *Salmonella*. However, the combination of 25 ppm LAE with 0.025% carvacrol or 50 ppm LAE with 0.025% carvacrol reduced *Salmonella* inoculum to undetectable level. Similarly at 22°C; 6 ppm, 12 ppm and 25 ppm LAE or 0.006%, 0.012% or 0.025% carvacrol did not result in any appreciable reductions in *Salmonella*. However, the addition of 6 ppm LAE with 0.025% carvacrol reduced the *Salmonella* counts by 4.4 log CFU/ml in comparison to the control while the combined treatment of 12 ppm LAE with 0.025% carvacrol or 25 ppm LAE with 0.006% or 0.012% carvacrol reduced the *Salmonella* inoculum to undetectable levels.

4.3.3 Reduction of *Salmonella* spp. and total microbial load in ground turkey of different fat percentage by lauric arginate and carvacrol

Fat content in ground turkey samples had significant influence on the concentrations of LAE and carvacrol that were required to inactivate *Salmonella* (Figures 4.1, 4.2 and 4.3). In general, proportionally higher concentrations of LAE and carvacrol were needed with increasing fat content in ground turkey samples. Treatment with 200 ppm or 1000 ppm LAE were insufficient to yield any reductions in *Salmonella* counts in 1%, 7% and 15% ground turkey samples, while 5000 ppm of LAE concentrations resulted into non-detectable *Salmonella* cell counts (minimum detection limit 1 log CFU/g) in all three turkey samples with 1%, 7% or 15% fat. At 2000 ppm, *Salmonella* reductions were dependent on the fat content of turkey samples. For example there was a ~4 log reduction in *Salmonella* counts in 1% fat ground turkey whereas there was only a 1 log CFU/g reduction in *Salmonella* counts in 7% and 15% ground turkey.

Effect of ground turkey fat content on the antimicrobial activity was more pronounced with carvacrol treatments. At 1% carvacrol, the *Salmonella* cell count decreased to undetectable levels in 1% fat ground turkey samples but it resulted in only a 0.5% log CFU/g reduction in 7% fat ground turkey samples, while no reductions in *Salmonella* counts were observed for 15% fat ground turkey samples. At 2% carvacrol treatment, there were about ~2-3 log CFU/g reductions in *Salmonella* cell counts in both 7% and 15% ground turkey samples. For 7% fat ground turkey samples, 5% carvacrol concentrations were needed to reduce the *Salmonella* cell counts to an undetectable level whereas the same 5% concentration of carvacrol in 15% fat ground turkey samples yielded only ~4 log reductions in *Salmonella* cell count. In the case of total microbial loads, both 2000 and 5000 ppm LAE treatment resulted in ~3 log CFU/g reductions compared to ~ 5 logs CFU/g total bacterial load detected in control samples. With

carvacrol, the reductions in total microbial counts were dependent on both carvacrol concentrations and fat content in ground turkey samples. Increasing carvacrol concentrations resulted in proportionally higher reductions in *Salmonella* cell counts for all 1%, 7% and 15% ground turkey samples. Treatment with 1% carvacrol resulted in a ~2 log CFU/g reduction in the total microbial count for 1% ground turkey samples whereas the 1% carvacrol treatment did not result in any appreciable reductions in the total microbial count for 7% and 15% ground turkey samples. At 2% carvacrol, the reductions in total microbial counts were ~3.7, 3.6 and 1.7 log CFU/g for 1%, 7% and 15% ground turkey samples, respectively. Treatment with 5% carvacrol reduced the total microbial count by ~4 log CFU/g in 1% and 7% ground turkey samples and ~2.5 log CFU/g in 15% ground turkey samples.

4.3.4 Lauric arginate and carvacrol show synergistic effect against three strain mixture of *Salmonella* spp. in ground turkey

Figure 4.4 shows the reductions in mixed strain *Salmonella* count by an antimicrobial mixture of LAE and carvacrol. LAE concentration of 200 ppm and 2000 ppm or carvacrol concentrations of 1% were selected based on the prior experiments in which these compounds when applied individually did not yield any measurable reductions in *Salmonella* counts in 7% ground turkey samples (Figure 4.4). The combination of 200 ppm LAE with 1% carvacrol did not result in any synergistic action to reduce *Salmonella* counts; this combined treatment did not vary from that of control samples (Figure 4.4). However, when LAE at 2000 ppm was mixed with 1% carvacrol, there was a synergistic action as *Salmonella* counts reduced to less than 1 log CFU/g from 7% fat ground turkey samples from the initial challenge load of 5 log CFU/g.

4.4 Discussion

In this study, we evaluated the antimicrobial efficacy of LAE and carvacrol on ground turkey samples. In the initial experiments, the temperature dependent effect of these antimicrobials and synergistic interaction between these antimicrobials against inactivation of a three strain mixture of *Salmonella* was evaluated. The antimicrobial activity of LAE is based on the disruption/instability of the plasma membrane lipid bilayer (Rodriguez and others 2004). Similarly, essential oils containing the active constituent carvacrol are proposed to interact with the lipid bi-layer of cytoplasmic membranes which results into damage and loss of integrity of the cell membrane with a subsequent leakage of the cellular material from inside the cells (Ultee and others 1999; Burt, 2004). For both LAE and carvacrol, our study revealed that antimicrobial efficacy of these compounds is dependent on temperature. Previously, Veldhuizen and others (2007) reported that 2.5 mm of carvacrol was able to reduce L. monocytogenes population by 4 log CFU/ml at 30°C while the very same carvacrol concentration was completely ineffective at 10°C. At a high temperature, the membrane fluidity of the bacterial cell increases which may allow easier transfer of antimicrobials within bacterial cells. In our broth assay, we included 22°C temperature because it allowed the simulation of turkey processing environment. Also, prior studies showed that the optimal biofilm formation in *Salmonella* occurs at around 20°C and biofilm cells present in food processing plant environment may be the major cause of contamination in the food processing (Giaouris and others 2005; Stepanovic and others 2003)

LAE concentrations between 50 ppm and 200 ppm and carvacrol concentrations between 0.05% and 0.1% were sufficient for the inactivation of 6 log CFU/ml of three strain mixtures of *Salmonella* in a broth model at 4°C, 22°C and 45°C. However, the

minimum concentrations of these antimicrobials needed against *Salmonella* spp. inactivation in ground turkey were at least 10 to 25-fold higher. Previously, numerous other studies have reported that the level of antimicrobial concentrations effective in any food system is typically 10 to 100-fold greater than those required in broth studies (Burt, 2004). Though the exact mechanism for the decreased antimicrobial efficacy in the food system is not well understood, the product complexity and composition is likely to be a major determinant. Some of the other possible reasons for such behavior include the greater availability of nutrients in a food substrate that can aid in the repair of damaged cells (Gill and others 2002); a lower water content in a food substrate compared to the broth media which can limit the access of antimicrobial to bacterial cells; and the ability of intrinsic factors such as fat, protein, carbohydrate, salt content and antioxidants that can neutralize the antimicrobials (Smith-Palmer and others 2001). Moreover, results from this study also showed substrate dependent effect against *Salmonella* inactivation. With the 200 ppm LAE treatment, there was about a 4 log CFU/g reduction in *Salmonella* counts in 1% fat ground turkey while the treatment of 7% and 15% ground turkey with the very same LAE concentrations only reduced the *Salmonella* counts by 1 log CFU/g. Similarly, 1% of carvacrol was enough to reduce the *Salmonella* count to an undetectable level in 1% ground turkey while the same carvacrol concentrations had a limited effect in 7% and 15% ground turkey meat. Since each of the turkey samples received a fixed 1 ml solution for antimicrobial delivery in which the higher antimicrobial concentrations were able to reduce *Salmonella* to undetectable level, the reduced efficacy appears to be function of the substrate rather than the inadequate distribution of the antimicrobials in ground turkey samples. Our previous study determining the efficacy of sublethal 400 ppm LAE concentration in skim milk and whole milk showed that the higher L.

monocytogenes growth in whole milk compared to the skim milk in which the fat concentration appearing to be one of the contributing factors that reduced LAE efficacy (Soni and others 2010). In terms of the product composition based on the product label, 1% fat ground turkey contained 23.2% protein (24.2% total solid), 7% fat ground turkey contained 17.9% protein (26.0% total solid) and 15% fat ground turkey contained 16% protein (31.4% total solid) with no carbohydrate present in any turkey samples and fat and protein representing >99% total solid content. The reduced efficacy of the LAE or carvacrol in higher fat containing ground turkey samples could be a function of the total solid content. However, the influence of fat concentration seems more pronounced since the variations in the total solid contents are not of large magnitude. Previously, Veldhuizen and others (2007) reported that albumin was able to bind to the carvacrol and consequently >0.2% of bovine serum albumin and egg yolk inhibited carvacrol activity. In our assay, LAE and carvacrol were much more effective in 1% fat ground turkey which contained a higher protein content compared to other samples. Hence, our findings suggest that in addition to the protein concentration as observed by Veldhuizen and others, (2007), the fat portion could also decrease the efficacy of antimicrobials and the mechanism of action needs to be further investigated.

Lauric arginate is relatively new FDA approved food preservative at concentrations up to 200 ppm of product weight (USFDA, 2005). With respect to determining the efficacy of LAE, only a limited numbers of studies have been performed so far and the majority of these studies focused on L. monocytogenes as a targeted organism. These studies indicated that the effect of LAE is short term with a maximum bactericidal activity observed within the first 24 h against the targeted pathogen while remaining bacteria that were able to survive and proliferate during long term storage

(Luchansky and others 2005; Taormina and Dorsa, 2009b; Martin and others 2009). Moreover, the majority of these studies have been performed on the intact food substrate such as an intact whole ham, frankfurter or cheese blocks (Luchansky et al., 2005; Taormina and Dorsa, 2009a; Taormina and Dorsa, 2009b; Soni and Nannapaneni, 2010). However, the results from our study indicate that currently approved 200 ppm LAE when applied internally as part of a product formulation in ground turkey is not sufficient to meaningfully reduce the *Salmonella* counts. The bactericidal concentrations of LAE observed in a broth model is in the range of between 12 ppm to 50 ppm for L. monocytogenes (Soni and Nannapaneni, 2010; Brandt and others., 2010). Also in both skim and whole milk samples, the FDA approved 200 ppm LAE failed to show any reductions in L. monocytogenes counts during 15 day sampling period (Soni and Nannapaneni, 2010). In the past, single antimicrobials were routinely used for controlling food safety associated risks. However, recently the focus has shifted to identifying the ideal mixtures of different antimicrobials that can provide a broad spectrum of antimicrobial activity while reducing the need for higher antimicrobial concentrations (Neetoo and others 2008). In this study, we observed a synergistic action between sublethal concentrations of LAE and carvacrol when used in a combination. Previously, a mixture of LAE and nisin was found to produce additive effect against L. monocytogenes cells (Brandt and others 2010). In other reports, the combinations of essential oil constituent thymol when mixed with citric acid exerted a synergistic action against S. Typhimurium (Nazera and others 2005). We also observed a synergistic effect between LAE and carvacrol in 7% fat ground turkey samples. The individual treatment of LAE even at 2000 ppm or carvacrol at 1% did not result in any appreciable reductions in mixed strain *Salmonella* counts; however, when combined together, they reduced the

Salmonella counts to less than 1 log CFU/g in 7% ground turkey samples from the 5 CFU/g of initial load.

4.5 Conclusions

In conclusion, our findings show that the essential oil constituent carvacrol in combination with LAE has a strong synergistic antimicrobial activity against the three strain mixture of *Salmonella* spp. in ground turkey samples. Our findings also show that the antimicrobial efficacy of carvacrol and LAE were strongly influenced by the percentage fat in the ground turkey products. Further studies should be conducted to understand the mechanisms of synergistic activity between carvacrol and LAE against *Salmonella* spp. Also, future studies are needed to evaluate the combined effect of these GRAS antimicrobial treatments during the heating step to achieve a complete elimination of *Salmonella* in cooked poultry products.

Table 4.1 Effect of temperature on the antimicrobial efficacy of lauric arginate and carvacrol against three strain mixture of *Salmonella* spp. in tryptic soy broth

Antimicrobial		*Salmonella* spp. (log CFU/ml)		
	Concentration	4°C	25°C	45°C
Lauric arginate	0 ppm	5.7 ± 0.1	5.9 ± 2	5.7
	25 ppm	5.7 ± 0.2	4.7 ± 0.6	3.3 ± 0.4
	50 ppm	5.5 ± 0.4	ND	ND
	100 ppm	2.1 ± 0.3	ND	ND
	200 ppm	ND	ND	ND
Carvacrol	0%	5.7 ± 0.4	5.8 ± 0.2	5.7 ± 0.7
	0.025%	5.6 ± 0.2	5.7 ± 0.5	4.3 ± 2
	0.05%	2.6 ± 0.1	ND	ND
	0.1%	ND	ND	ND
	0.2%	ND	ND	ND

Note: ND = Not detected. Minimum detection limit is 1log CFU/m

Table 4.2 Synergistic interactions of lauric arginate and carvacrol at two temperatures on the reduction of three strain mixture of *Salmonella* spp. in tryptic soy broth.

Temperature	Antimicrobial treatments	*Salmonella* spp. (log CFU/ml)
4°C	Control	5.6 ± 0.4
	25 ppm LAE	5.4 ± 0.2
	50 ppm LAE	5.1 ± 0.1
	0.025% carvacrol	5.1 ± 0.3
	25 ppm LAE + 0.025% carvacrol	ND
	50 ppm LAE+ 0.025% carvacrol	ND
22°C	Control	6 ± 0.1
	6 ppm LAE	5.7 ± 0.2
	12 ppm LAE	5.6 ± 0.1
	25 ppm LAE	4.8 ± 0.2
	0.006% carvacrol	5.8 ± 0.2
	0.012% carvacrol	5.5 ± 0.2
	0.025% carvacrol	5.3 ± 0.2
	6 ppm LAE + 0.025% carvacrol	1.3 ± 0.7
	12 ppm LAE + 0.025% carvacrol	ND
	25 ppm LAE + 0.006% carvacrol	ND
	25 ppm LAE + 0.012% carvacrol	ND

Note: ND = Not detected. Minimum detection limit is 1log CFU/ml

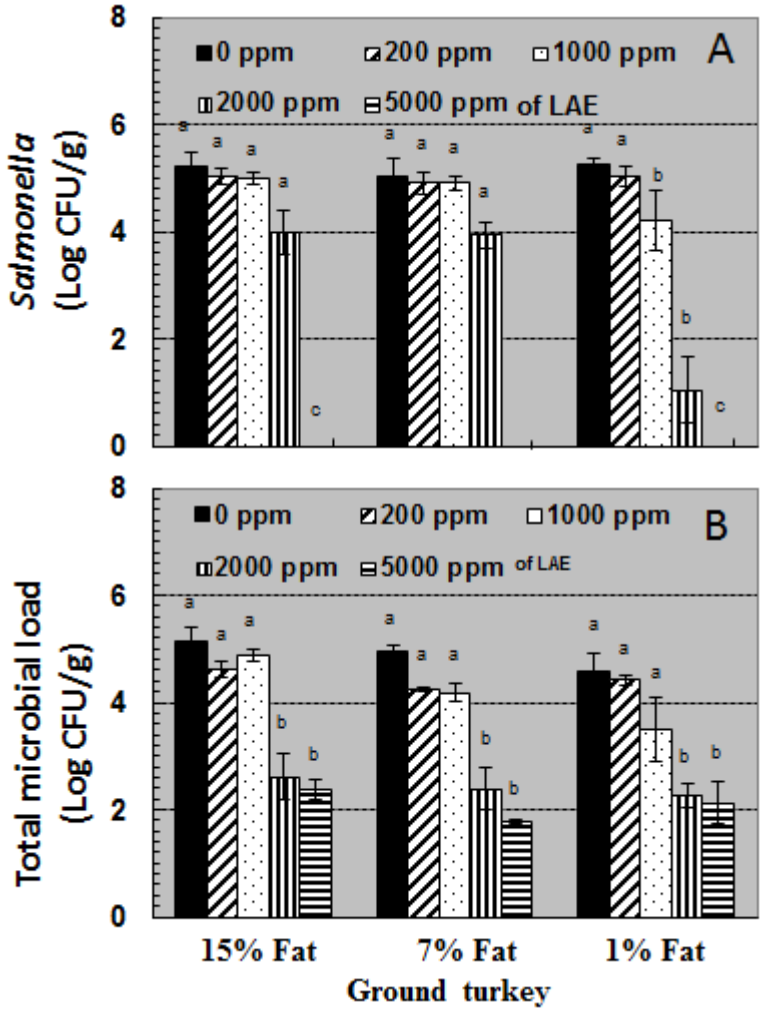

Figure 4.1 Efficacy of different concentrations of lauric arginate (0 to 5000 ppm) in ground turkey containing 15%, 7% and 1% fat at 4°C against (A) three strain mixture of *Salmonella* spp.; and (B) total microbial load.

Notes: Bars with different lowercase letter show the mean significant differences for *Salmonella* reduction by different essential oils at varying concentrations based on one-way ANOVA posthoc tukey test ($P \leq 0.05$).

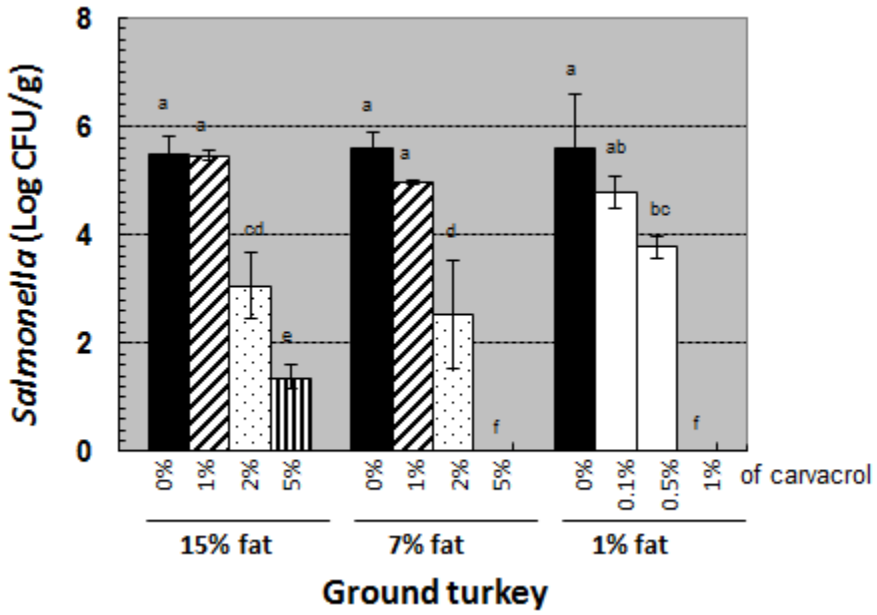

Figure 4.2 Efficacy of different concentrations of carvacrol (0 to 5%) in ground turkey containing 15%, 7% and 1% fat at 4°C against three strain mixture of *Salmonella* spp.

Note: Bars with different lowercase letter show the mean significant differences for *Salmonella* reduction by different essential oils at varying concentrations based on one-way ANOVA posthoc tukey test ($P \leq 0.05$).

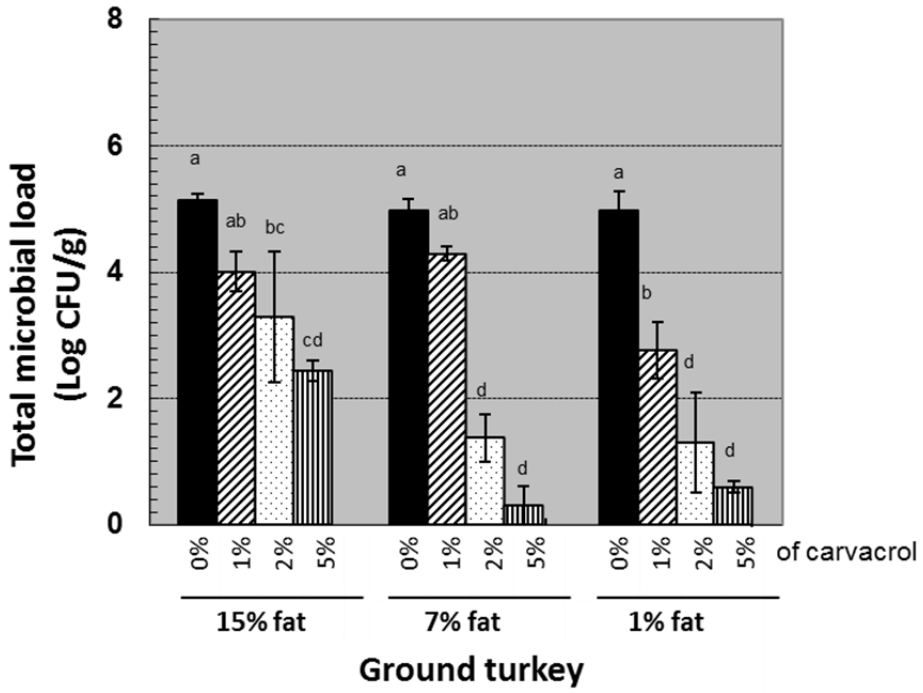

Figure 4.3 Efficacy of different concentrations of carvacrol (0 to 5%) in ground turkey containing 15%, 7% and 1% fat at 4°C against total microbial load

Notes: Bars with different lowercase letter show the mean significant differences for *Salmonella* reduction by different essential oils at varying concentrations based on one-way ANOVA posthoc tukey test ($P \leq 0.05$).

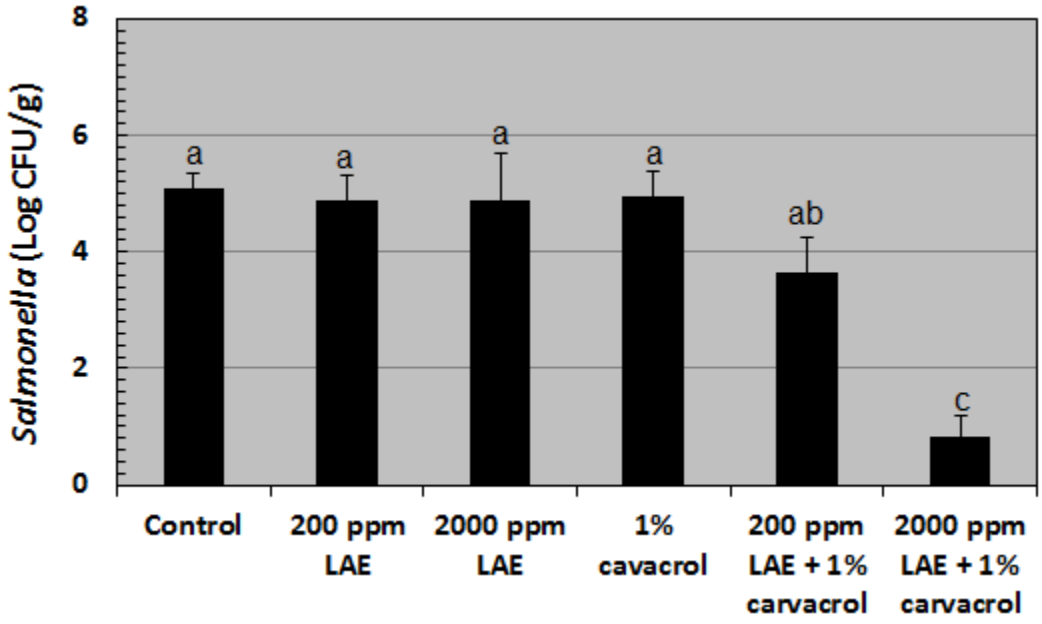

Figure 4.4 Synergistic effect of carvacrol and lauric arginate on the reduction of three strain mixture of *Salmonella* spp. in ground turkey containing 7% fat

Notes: Bars with different lowercase letter show the mean significant differences for *Salmonella* reduction by different essential oils at varying concentrations based on one-way ANOVA posthoc tukey test ($P \leq 0.05$).

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CHAPTER V

SUMMARY AND CONCLUSIONS

The essential oils of thyme, oregano and carvacrol showed effectiveness against *Salmonella* cells in planktonic and biofilm forms. The present study revealed that essential oils of thyme, oregano and carvacrol showed minimum inhibitory and bactericidal effect against *Salmonella* spp at 0.025%. A sublethal concentration of 0.06% essential oils of thyme, oregano and carvacrol were effective in reducing the biofilm forming capability of *Salmonella* spp. in polystyrene 24-well microtiter plates. When the essential oils of thyme, oregano and carvacrol were used at 0.05% or 0.1%, they were highly effective in killing the preformed biofilms of mixed strains of *Salmonella* spp. on polystyrene and stainless steel surfaces. When we examined the bactericidal effect of carvacrol and LAE individually and in combinations in tryptic soy broth (TSB), we found out that carvacrol at 0.025% in combination with 25 ppm to 50 ppm of LAE yielded a 5 log CFU/ml reduction of *Salmonella* within 30 min at 4°C. However, 0.025% carvacrol, 25ppm or 50ppm LAE showed no *Salmonella* reduction under the same conditions. In ground turkey experiments, there was no significant reduction in *Salmonella* with 1% carvacrol or 2000 ppm LAE when applied individually. On the other hand, when these two antimicrobial concentrations were mixed together in ground turkey samples containing 7% fat, they elicited a synergistic effect by yielding a reduction of 5 log CFU/g of *Salmonella* counts. Further studies will look into the long term storage of ground turkey samples stored at 4°C containing these combinations of GRAS

antimicrobials and will evaluate any organoleptic qualities associated with the use of essential oils in these retail poultry products. Since both LAE and carvacrol showed increased efficacy in reducing *Salmonella* cell counts at higher temperatures, the synergistic action of these antimicrobials in combination with cooking steps should be investigated for the poultry products that are of high risk of *Salmonella* contamination.