Combinations of multiple natural antimicrobials with different mechanisms as an approach to control *Listeria monocytogenes*

A Thesis Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Savannah Grace Hawkins
August 2016

ACKNOWLEDGEMENTS

I would first like to thank my major professor Dr. P Michael Davidson for accepting me as a student in his lab not only as an undergraduate but graduate student. I could not have asked for a better mentor who I also consider a friend. His patience, encouragement, and sarcastic comments when I have been overwhelmed with work have kept me motivated each and every day. I am so very grateful that he mentored me when retirement was right around the corner. I would also like to thank my committee members, Dr. David Golden and Dr. Faith Critzer. Their continuous support by answering any questions or concerns has eased my daily struggles throughout my master's research.

I am deeply thankful for the support that every member of the Food Science Department has given me throughout this journey. Dr. Bozkurt, Dr. Monu, Dr. Chen, and Dr. Techathuvanan thank you all for your patience from the very beginning in teaching me the basics of food microbiology. To my lab mates, thank you for keeping the working environment fun and providing advice along the way.

Finally, thank you to all my friends and family. The love and support each of you showed me through answering my many phone calls, weekend visits, and snail mail cannot be matched. My mom has been the best cheerleader I could ask for, and my dad has kept me levelheaded with his words of wisdom. My sisters, Tori and Cara, the positive attitude the two of you provided for any predicament brightened any day.

ABSTRACT

To improve food safety and shelflife requires the use of preservation processes, such as physical (heat, refrigeration) or chemical (antimicrobial addition) processes. Regulatory approved synthetic food antimicrobials (preservatives) have some uses but are very limited in their spectrum of activity. Thus, alternatives are needed to conventional chemical antimicrobials. One method is to use naturally occurring antimicrobials, especially those found in spices and herbs, essential oils (EO) and essential oil components (EOC). EOs have been shown to have antimicrobial activity but the activity is highly variable. Finding a combination of EOs, EOCs, or other natural antimicrobials that act synergistically would allow a reduction in the use concentration. This is important for EO as they may contribute undesirable sensory effects to foods. To achieve synergistic interaction of antimicrobials likely requires that individual compounds have different mechanisms of inhibition or inactivation. Therefore, the objective of this study was to attempt to achieve synergistic antimicrobial interactions and reduce use concentrations by combining EOs and a naturally occurring hydroxycinnamic acid with reported different mechanisms. Oregano essential oil (OEO), basil essential oil (BEO), coriander essential oil (CEO), and ferulic acid (FA) were evaluated alone and in combination against *Listeria monocytogenes* at pH 6.0 and 25°C for 48h. A broth dilution assay was used to determine the minimum inhibitory concentrations (MIC) of individual and combined antimicrobials. Fractional inhibitory concentrations (FIC) were calculated and the

interactions interpreted as synergistic (FIC ≥0.5), additive (FIC >0.5 and <1.5), or antagonistic (FIC ≤1.5). MICs of compounds alone against *L. monocytogenes* Scott A were 250 ppm (parts per million) OEO, 2500 ppm CEO, 7500 ppm BEO, and 5000 ppm FA. Combinations of OEO+BEO, CEO+BEO, CEO+FA, BEO+FA, OEO+CEO+BEO, BEO+CEO+FA, and OEO+BEO+FA, and OEO+CEO+BEO+FA resulted in synergistic inhibition of *L. monocytogenes* (FIC ≤ 0.5). The quaternary combination of OEO+CEO+BEO+FA was inhibitory at 31.25, 312.5, 937.5, and 625 ppm, respectively. Combining natural antimicrobials with suggested different mechanisms may be a solution for controlling foodborne pathogens and reducing use concentrations. A quaternary antimicrobial blend reduced the concentration of each compound needed for inhibition by 87.5% which could also reduce the potential for negative sensory effects.

TABLE OF CONTENTS

CHAPTER I
INTRODUCTION 1
CHAPTER II
LITERATURE REVIEW 4
Foodborne pathogenic bacteria 4
Listeria monocytogenes 5
Antimicrobial food preservation 8
Natural antimicrobials 9
Plant Derived Antimicrobials 11
Phenolic Compounds 12
Hydroxycinnamic acid derivative ferulic acid 13
Essential oils and their mechanism of antimicrobial action 15
Combining natural antimicrobials to increase antimicrobial activity 18
Oregano 22
Coriander 24
Basil 26
Applications of EOs to foods as antimicrobials 29
CHAPTER III
MATERIALS AND METHODS 31
Antimicrobials *31*
Bacteria *31*
Determination of minimum inhibitory concentrations (MICs) *32*
Determination of combined antimicrobial effects *32*
FIC calculation *33*
CHAPTER IV
RESULTS AND DISCUSSION 35
MICs of single antimicrobials 35
Efficacy of antimicrobial combinations 37
Binary antimicrobial combinations 38
Tertiary antimicrobial combinations *41*
Quaternary antimicrobial combinations *43*
CHAPTER V
CONCLUSIONS AND RECOMMENDATIONS 47
LIST OF REFERENCES 49
APPENDIX 57
VITA 65

LIST OF TABLES

Table 1. Design of Antimicrobial Combination Tests with Fractional Inhibitory Concentration Index and Interpretation of Effect.....58
Table 2. MICs of Antimicrobials against *Listeria Monocytogenes* Scott A at pH 6.0 And 25°C 60
Table 3. Effect of Binary Combinations on *Listeria monocytogenes* Scott A at pH 6.0 and 25°C.....61
Table 4. Effect of Tertiary Combinations on *Listeria monocytogenes* Scott A at pH 6.0 and 25°C 62
Table 5. Effect of Tertiary Combinations on *Listeria monocytogenes* Scott A at pH 6.0 and 25°C 63
Table 6. Interpretation of Effects of Combined Antimicrobials against Listeria monocytogenes Scott A at pH 6.0 and 25°C 64

CHAPTER I

INTRODUCTION

Listeria monocytogenes is a pathogenic bacterium which can cause a life-threatening illness called listeriosis. The bacterium is able to survive and grow anaerobically or aerobically at refrigeration temperatures on food products *(27)*. Transmission of *L. monocytogenes* is primarily associated with contaminated ready-to-eat (RTE) foods, both prepackaged and packaged at retail, such as meat, seafood, vegetables, and pasta salads *(49)*. According to the US Food and Drug Administration (FDA), *L. monocytogenes* is a “zero-tolerance” pathogen in cooked, RTE foods meaning the detection of the bacterium in a RTE food item makes that product adulterated *(79)*. Therefore, methods to inhibit the growth of or inactivate *L. monocytogenes* in RTE foods are imperative to enhance food safety.

Improving safety and quality shelflife of food requires the use of preservation processes, such as physical (e.g., heat, refrigeration) or chemical (e.g., antimicrobial addition) processes. Regulatory-approved synthetic food antimicrobials (preservatives) have some uses, but are limited in their overall spectrum of antimicrobial activity and are generally inactive at the pH of low-acid foods. Thus, alternatives are needed to conventional chemical antimicrobials. One method suggested for control of microorganisms in foods has been application of naturally occurring antimicrobials, especially those found in spices and herbs including essential oils (EO) and essential oil components (EOC) *(17)*.

Many of these compounds have a broad spectrum of inhibition or inactivation against gram-positive bacteria, gram-negative bacteria, yeasts, and molds *(97)*. EOs have been studied extensively for their ability to prolong shelflife and inhibit the growth of pathogenic microorganisms *(43)*. However, a drawback to the addition of EOs to food products is their sensory impact. EOs are aromatic and volatile so application may negatively affect food taste and/or odor. To overcome this drawback, a reduction in the use concentration of EOs and EOCs has been sought along with the use of other plant derived, often phenolic-based antimicrobials (e.g., hydroxycinnamic acids) *(25)*. To reduce the concentrations used, studies have focused on using combinations of EOs, EOCs and plant extracts to minimize concentrations and reduce sensory effects *(32)*. Combining these compounds can lead to one of three interactions, synergistic, additive, or antagonistic effects *(19)*. Finding combinations which are synergistic will likely require the antimicrobial compounds to have different mechanisms of inhibition or inactivation targeting multiple biochemical processes *(35)*.

The objective of this study was to combine EOs and a naturally occurring phenolic plant extract of the hydroxycinnamic acid class having reportedly different antimicrobial mechanisms to target synergistic interactions. The EOs used included oregano (OEO), coriander (CEO), basil oil (BEO), and the hydroxycinnamic acid used was ferulic acid (3-methoxy-4-hydroxycinnamic acid; FA). They were evaluated alone and in all combinations against *L. monocytogenes*. OEO (*Origanum vulgare*) is comprised of carvacrol (2-methyl-5-

(1-methylethyl)-phenol), thymol, γ-terpinene, and ρ-cymene *(10)*. Carvacrol has been shown to interfere with flagellar function of *Campylobacter jejuni (2)*. Other reported mechanisms for carvacrol and thymol include disruption of the bacterial cell membrane to increase passive permeability *(46)*. Similar to OEO, CEO (*Coriandrum sativum L.)* antimicrobial activity is reported to be primarily due to cytoplasmic membrane damage *(83)*. There have also been literature reports that CEO has the ability to chelate transition metals *(92)*. BEO (*Ocimum basilicum*) is highly variable in its composition depending on geographic location. Antimicrobial activity reported for BEO containing high concentrations of linalool (3,7-dimethyl-1,6-octadien-3-ol) and eugenol (4-allyl-2-methoxyphenol) led to cell leakage in both gram-negative and gram-positive bacteria *(6, 40)*. FA is a naturally occurring hydroxycinnamic acid ester *(25)*. Undissociated FA (pKa = 4.42) has been shown to cross the plasma membrane into the bacterial cytoplasm where it can dissociate releasing H^+ ions causing the internal pH of the bacterial cell to decrease. This reduction in pH may affect various metabolic pathways but its antimicrobial activity is mainly attributed to its ability to inhibit ATPase activity of a cell *(16, 57, 76)*. Because OEO, CEO, BEO and FA have slightly different reported mechanisms, they were selected as potential candidates for targeting synergistic interactions.

CHAPTER II

LITERATURE REVIEW

Foodborne pathogenic bacteria

Pathogenic bacteria in foods are one of the major concerns when striving to ensure the safety of the food supply. In the United States, approximately 48 million foodborne illnesses are attributed to major foodborne pathogens annually *(78)*. Of these cases, 128,000 require hospitalizations and 3,000 result in death. Foodborne illness occurs when a food is contaminated by a pathogenic microorganism and the food is ingested by a susceptible individual. Recent widely reported foodborne illness outbreaks have been attributed *Escherichia coli, Salmonella*, and *Listeria monocytogenes*. Scallan et al. *(78)* estimated that Shiga toxin-producing *E. coli* O157:H7 and non-Shiga toxin-producing *E. coli* O157:H7 result in approximately 175,000 illnesses and 20 deaths, non-typhoidal *Salmonella* species result in 1.0 million illnesses and 400 deaths, and *L. monocytogenes* is estimated to cause 1,600 illnesses and 255 deaths annually in the United States.

Identifying food products contaminated by pathogenic bacteria that may cause illness is difficult for several reasons. Infectious doses of foodborne pathogens can range from 10^1-10^6 cells. Detecting this small number of microorganisms in foods is complex and often time consuming. Some bacteria have the ability to produce toxins (intoxications) that can cause illness. Therefore lack of detection of these toxin-producers does not indicate a safe product *(4)*.

Finally, pathogenic bacteria often do not cause changes in the sensory properties of foods and are therefore impossible to detect using aroma changes *(101)*.

The sources of pathogens are animals and animal fecal material, water, air, soil, and humans. Within the food supply chain, contamination or cross contamination can occur by improper pre- or post-harvest handling of food products or through inadequate sanitation associated with processing. Growth of pathogens may occur during improper storage conditions. The type of food product affects pathogenic growth. Its micro-architecture can range from liquid, gel, oil-in-water emulsion, water-in-oil emulsion, gelled emulsion, or solid surface. Microorganisms can grow within the liquid of foods allowing motility or as colonies or biofilms on food surfaces. Food products supply the necessary nutrients to aid in microbial growth *(101)*.

Listeria monocytogenes

L. monocytogenes is a gram-positive, rod shaped, microaerophilic, non-sporeforming bacterium. The organism is catalase positive and oxidase negative. On blood agar*, L. monocytogenes* produces β-hemolysis. Studies have shown *L. monocytogenes* grows well in tryptic soy broth supplemented with 0.6% yeast extract or brain heart infusion broth at an optimum pH from 5.0-9.0 *(27)*. *L. monocytogenes* has the ability to grow over a temperature range between 0-42°C. Optimum temperature for growth is 30-35°C, however the organism is characteristic for its ability to grow at low temperatures between 0-8°C *(7)*. At

temperatures between 20 and 25°C, peritrichous flagella are produced and assembled on *L. monocytogenes*' cell surface giving it a tumbling motility *(66)*.

Listeria monocytogenes was first recognized as an animal pathogen in 1926. However, its significance as a human foodborne pathogen was not noted until approximately 30 years ago *(7)*. Consumption of food contaminated with *L. monocytogenes* may cause the illness listeriosis. In the early 1980's, outbreaks of human listeriosis, often associated with dairy products, led to severe illnesses and high mortality rates among immunocompromised individuals shedding light on the severity of the disease *(7)*. Foodborne listeriosis is estimated to have a 15.9% fatality and 94% hospitalization rate *(78)*. The majority of human cases are due to suppressed immune systems. The most susceptible individuals are neonates and the elderly, cancer and immunosuppressive therapy patients, and individuals with AIDS *(77)*. Adult listeriosis can be divided into two phases, enteric and invasive. The enteric phase occurs 1-2 days after consumption of food contaminated with *L. monocytogenes*. Symptoms are flu-like with occasional diarrhea. The invasive phase is much more severe with the possibility of sepsis, meningitis, and endocarditis. Early diagnosis and treatment with antibiotics and fluid replacement have shown to be successful. High fatality rates of up to 70% are seen if listeriosis is untreated. As previously mentioned, fetuses are extremely susceptible to listeriosis leading to abortion, stillbirth, premature birth, or meningitis in the newborn *(13)*.

Foodborne transmission of *L. monocytogenes* was first identified in 1981. Previously the only documented cause of human listeriosis were through direct infection from an infected animal to a cutaneous lesion. Foodborne transmission was first documented in Nova Scotia, Canada between March and September of 1981. A case-control study concluded the 41 cases of listeriosis were due to consumption of *L. monocytogenes* contaminated cabbage made into coleslaw and served at a restaurant *(27, 86)*. Since that initial outbreak, the organism has been isolated from dairy, meat, egg, produce, and seafood products including processed foods *(27)*. A recent multistate outbreak of listeriosis was linked to contaminated ice cream (Blue Bell Creameries, Brenham, TX). It was found that the ice cream products were being manufactured under conditions and controls that enabled microbial growth *(68)*.

L. monocytogenes survival in various food items can be attributed to its ability to adapt to low pH and temperatures *(27, 28)*. Food products with a high pH, moisture, and nutrient content are ideal for listerial growth. This food composition aids in *L. monocytogenes* growth at low temperatures including refrigeration *(13, 27)*.

Food processors have utilized many processing and preservation methods to eliminate, inactivate, or inhibit the growth of *L. monocytogenes*. Thermal processing at 62.7°C for 1.0 min will inactivate *L. monocytogenes* in whole milk *(24)*. Drying and the addition of salt or sugar during processing lower the water activity (a_w) of foods to extend shelf life. *L. monocytogenes* can tolerate both high

salt concentrations and low a_w. The organism has the ability to grow at an a_w greater than 0.90 and can survive at a a_w less than 0.90 *(45, 48)*. In a commercial cheese brine (23.8% NaCl, pH 4.9) stored at 4°C, *L. monocytogenes* was isolated after 259 days of storage *(48)*. The addition of synthetic antimicrobials to food products during processing has been shown to inhibit the growth of *L. monocytogenes*. Acid dips of 0.25% sodium diacetate and 1.8% sodium lactate for two minutes inhibited the growth of *Listeria* on turkey frankfurters stored at 10°C *(5)*. Sodium diacetate and sodium lactate in combinations are often used in RTE comminuted meat products to control the growth of *L. monocytogenes (31)*. The addition of 2.5% liquid smoke flavoring to meat products has also been shown to inhibit the growth of *L. monocytogenes* stored at 4°C *(59)*.

Antimicrobial food preservation

Food preservation is constantly evolving to improve food quality, safety, and storage. Traditional food preservation dates back to prehistoric times, primarily with the physical processes such as heating, cooling, drying, and fermentation. The addition of certain chemical compounds, such as salt, nitrites, and sulfites, were adjuncts to the physical processes. Today, more than 2,500 chemical additives exist to aid in various stages of food processing. These chemical additives function as food preservatives, coloring and flavoring agents, nutritional additives, and texture enhancers, among others, in processed goods *(9)*. The addition of antimicrobial food preservatives enables global distribution

and decreased physical processing of foods *(71)*. Antimicrobial food preservation improves the safety and quality of foods by inhibiting or inactivating spoilage and pathogenic microorganisms *(18)*. The U.S. Food and Drug Administration (21 CFR 170.3 (o)(2)) states antimicrobials are “used to preserve food by preventing growth of microorganisms and subsequent spoilage, including fungistats, mold and rope inhibitors”. Antimicrobials may be added directly into a food product, in food packaging, on food contact surfaces, or in food processing environments as methods for food preservation *(17)*. Some antimicrobials exist naturally in foods or they can be added to a product in a synthetic form for preservation *(15)*.

It has been reported that consumers in the 2010s are desiring foods to be “less processed” and “less packaged” with fewer to no “preservatives” *(103)*. In addition, consumers want food that is high in nutrition, convenient to prepare, 100% safe, low priced, and environmentally friendly and sustainable *(71)*. Globalization has further altered the current state of food preservation. Location of production, processing, packaging, and storage often take place in different geographical areas. This length of time for products to reach retailers and consumers has thus been extended *(15)*. These circumstances have led to greater research on the use of natural antimicrobial substances as a replacement for synthetic chemical antimicrobials in food.

Natural antimicrobials

Interest in the use of naturally occurring antimicrobials has drastically increased in response to consumer demands for reduced use or elimination of

synthetic chemical preservatives *(17, 103)*. Natural antimicrobials can be used to preserve food by inhibiting or inactivating the growth of pathogenic or spoilage microorganisms. An ideal “label-friendly” or “clean label” natural antimicrobial would have antimicrobial activity when added as an unaltered product or as an extract with minor purification or refining *(17)*. Davidson et al. defines an ideal natural antimicrobial as one that would “(*a*) be effective at low concentrations in its natural form, (*b*) be economical at use levels, (*c*) cause no sensory changes to the product, (*d*) inhibit a wide array of pathogenic and spoilage organisms, and (*e*) be nontoxic”*(17)*. However, few, if any naturally occurring antimicrobials have all these properties *(17, 18)*.

Naturally occurring antimicrobials may be derived from animals, plants, or microorganisms. Most animal-derived antimicrobials likely evolved in animals, particularly vertebrates, as host defense mechanisms *(17)*. These antimicrobials are most commonly isolated from animal foods involved in immune response and/or as protection for neonates. For example, lactoferrin (iron-binding protein) and lysozyme (degrades bacterial cell walls) are present in bovine milk while poultry eggs contain ovotransferrin (binds iron) and lysozyme. These and other compounds have antimicrobial activity against bacteria, fungi, parasites, and viruses *(89)*. Plant-derived antimicrobials exist in over 1,340 different plant varieties *(100)*. The antimicrobials can be derived from a plant’s leaves, flowers, bulbs, rhizomes, or fruit. Antimicrobial activity is often related to plant defenses and can be found associated with compounds such as phenolics, terpenes,

aliphatic alcohols, aldehydes, ketones, acids, and isoflavonoids *(97)*. Microbially-derived antimicrobials are also used in by microorganisms for defense or protection. Bacteriocins which are inhibitory peptides produced by bacteria include nisin (*Lactococcus lactis* ssp. *lactis*). Nisin is active against spores produced by *Bacillus* and *Clostridium*. A second form of microbial control is the use of bacteriophages, or viruses that infect and lyse host bacteria. Bacteriophages are specific in target bacteria limiting their activity to a single species or strain *(17)*.

Plant Derived Antimicrobials

A wide variety of compounds produced in plants, herbs, and spices have been found to exhibit antimicrobial activity against bacteria, yeast, and mold. The biological function of these compounds aid in plant resistance to phytopathogens and insects in nature *(17)*. The natural antimicrobial systems that exist in plants include essential oils and organic acids *(97)*. These are secondary plant metabolites that are present in a plant but not necessary for growth or reproduction *(95)*. Simple and complex phenolic compounds have been identified as the most active plant-derived antimicrobial components and often occur in the essential oils *(81)*. Aldehydes, ketones, terpenes, isoflavonoids, aliphatic alcohols, and organic acids also contribute to antimicrobial efficacy *(97)*.

Essential oils are obtained from steam distillation, pressing, or solvent extraction of various parts of plants including leaves, seeds, flowers, and bulbs yield essential oils and extracts *(17)*. The *in vivo* concentration of plant-based

antimicrobials affects the antimicrobial efficacy. The same plant can vary in EO composition depending on geographic location, harvesting time, and extraction method *(97)*.

The food industry currently uses spices and herbs as flavoring agents. The majority of spices and herbs including their EOs are generally recognized as safe (GRAS) for that purpose *(97)*. Utilizing their antimicrobial properties to enhance the safety and preservation of foods therefore would meet consumer demands to replace synthetic food additives *(10)*.

Phenolic Compounds

A phenolic compound is a substance which possesses an aromatic ring with one or more hydroxyl groups *(34)*. Phenolic compounds from plants can be categorized into simple phenols and phenolic acids (hydroquinone, vanillin, p-cresol), hydroxycinnamic acid derivatives (ferulic, caffeic, and p-coumaric acid), and flavonoids (catechins, anthocyanidins, and flavons) *(39)*. Phenolic compounds have several functions within foods. For example, chlorogenic acid (3-(3,4-dihydroxycinnamoyl)quinic acid), a hydroxycinnamic acid derivative, participates in enzymatic browning in foods such as apples and pears *(26, 39)*.

Phenolic compounds also exhibit antimicrobial and antifungal activity *(16)* Simple phenolic compounds, such as phenol and cresols (methyl phenols), act as antimicrobials and flavor enhancers when deposited on cheese, meats, and fish during the smoking process *(20)*. Benzoic acid, proanthocyanidins, and flavonols from cranberries have been shown to inhibit *Saccharomyces bayanus*

(16, 54). Tannic acid, a polyphenolic present in the rinds and bark of plants, had antimicrobial activity against *A. hydrophila*, *E. coli*, *L. monocytogenes, S. Enteritidis, S. aureus*, and *S. faecalis (12, 16)*.

The strong antimicrobial activity of essential oils of plants is often likely due to their high percentages of phenolic compounds. Phenolic compounds are often amphiphilic which means that can interact with the fatty acids of the microbial cell membrane leading to disruption of electron flow, active transport, and proton motive force *(23)*. This polarity of phenolic compounds is due to its hydroxyl group. To demonstrate the importance of the hydroxyl group on a phenolic compound, Ultee et al. *(95)* compared the antimicrobial activity of a compound without a hydroxyl group, cymene to carvacrol, a compound with a hydroxyl group. Both carvacrol and cymene had an effect on membrane integrity of *B. cereus*. However, the presence of the hydroxyl group on carvacrol increased the leakage of K^+ and increased the influx of H^+ in *B. cereus*. Ultee et al. proposed that, “carvacrol acts as a transmembrane carrier of monovalent cations by exchanging its hydroxyl proton for another ion such as a potassium ion. Undissociated carvacrol diffuses through the cytoplasmic membrane” *(95)*.

Hydroxycinnamic acid derivative ferulic acid

Hydroxycinnamic acids occur naturally in foods as esters or glycosides *(38)*. The four natural states are coumaric, caffeic, ferulic, and sinapic acids *(25)*. Ferulic acid (4-hydroxy-3-methoxycinnamic; FA; CAS no. 1135-24-6) is naturally found as an ester cross-linked to polysaccharide in the cell wall of plants or less

commonly cross-linked with proteins. It exists at high concentrations in numerous plants including wheat bran, sugar-beet pulp, and corn kernel. Due to its low toxicity, FA is approved as a food additive for use in foods, beverages, and cosmetics *(63)*.

FA is a weak organic acid (pKa 4.42) *(42)*. Therefore, FA's antimicrobial activity is affected by the concentration of undissociated acid *(11, 76)*. FA is dissociated at pH levels above its pKa which decreases its ability to cross the cell membrane by passive diffusion *(57)*. Due to this, FA will have a higher antimicrobial activity at a pH close to its pKa. Miyague et al. *(57)* demonstrated this by testing FA at pH of 5.0, 6.0, and 7.0 against *L. monocytogenes.* The MIC of FA against *L. monocytogenes* in a broth dilution assay incubated for 48 h at 30°C increased with increasing pH from 2.5 mM (pH 5.0) to 5 mM (pH 6.0) to 10 mM (pH 7.0).

FA has been shown to inhibit the growth of bacteria, yeasts, and molds. Lyon and McGill *(53)* tested the antimicrobial activity of FA against *Erwinia carotovora* which may cause soft-rot of potatoes and other vegetables. Inhibition of *E. carotovora* was achieved with FA at 5,000 µg/ml in a nutrient broth *(16, 53)*. Antimicrobial activity was also noted by Herald and Davidson *(16, 37)* against both gram-positive and gram-negative bacteria in a broth dilution assay for 48 h at varying time intervals. FA inhibited the growth of *B. cereus* and *S. aureus* at 1000 µg/ml at pH 5.0 for 36 h and pH 6.0 for 9 hrs. The growth of *E. coli* was

inhibited at 500 µg/ml FA at pH 5.0 and 1000 µg/ml FA at pH 6.0 for 36 h *(16, 37)*.

The antimicrobial activity of FA is attributed to its ability to inhibit ATPase activity *(16, 57, 76)*. FA can cross the plasma membrane into the bacterial cytoplasm and once the compound dissociates, the internal pH of the bacterial cell decreases inactivating metabolic pathways. Rico-Munoz et al. *(76)* measured the effect of hydroxycinnamic acids on staphylococcal membrane-bound ATPase activity at 37°C. The ATPase activity was determined by measuring the liberation of phosphate in a 1 ml reaction mixture of 2.5 mM ATP, 150 mM KCl, and 50 mM Tris-acetate buffer for 30 min. FA (1200 mg/ml) inhibited the ATPase activity of *Staphylococcus aureus* at pH 6.0 *(76)*. Additionally, phenolic acids including FA increased the cellular membrane permeability of lactic acid bacteria. The effects of FA (2200 mg/ml) on *Oenococcus oeni* and *Lactobacillus hilgardii* membranes was studied by measuring the potassium efflux with a potassium-sensitive electrode, phosphate efflux determined by a Flow Injection System, proton efflux by extracellular pH monitoring, and cell membrane injury using a fluorochrome kit. Treatment with FA increased the phosphate, potassium, and proton efflux and decreased viable cell fluorescence indicating cellular membrane damage*(11)*.

Essential oils and their mechanism of antimicrobial action

Plant essential oils (EO) and their components (EOC) have antimicrobial activity against a wide range of microorganisms, including gram-positive and

gram-negative bacteria, yeasts, and molds. However, the extent of the activity varies with EO and with the spectrum of antimicrobial activity. The varying spectra can likely be attributed to the different EOC present in individual EOs, the variation in the activity of those components, and their interactions. EOs are composed of secondary plant metabolites that can be divided into four groups based on chemical structure: terpenes, terpenoids, phenylpropenes, and “others” *(41)*.

Terpenes are synthesized in the cytoplasm of plant cells and are composed of a hydrocarbon backbone. Monoterpenes ($C_{10}H_{16}$) and sesquiterpene ($C_{15}H_{24}$) are the primary terpenes. Examples include p-cymene and α-pinene *(41)*. The antimicrobial activity of terpenes is minimal to none. For example, Bagamboula et al. *(3)* found p-cymene had no antimicrobial activity against *S. sonnei* and *S. flexneri* at 85700 µg/ml using an agar well diffusion assay at 37°C for 24 hrs.

Terpenoids, the largest group of plant secondary metabolites, are composed of a hydrocarbon backbone with an oxygen molecule *(47)*. Terpenoids can be further categorized into alcohols, esters, aldehydes, ketones, ethers, phenols, and epoxides. Common terpenoids are carvacrol, linalool, and thymol *(41)*. The mechanism of antimicrobial action is proposed to be due to the hydroxyl group on these compounds. The hydroxyl group disrupts lipid bilayers in microbial cells, breaking down the structure thus increasing permeability *(43)*. It is then proposed that a leakage of metabolites and ions occurs *(10)*. A cell is able

to withstand a loss of cytoplasmic constituents without losing cell function but extensive loss can cause cell death *(23)*. Disruption of the cell membrane leads to disturbance of electron flow, active transport, and proton motive force *(23)*.

Phenylpropenes are synthesized from amino acids and are composed of a six-carbon aromatic phenol group and a three-carbon propene tail. Examples include eugenol, cinnamaldehyde, and vanillin *(41)*. Phenylpropenes can bind to and affect protein properties and inhibit the activity of enzymes such as ATPase, histidine, decarboxylase, amylase, and protease *(30, 41)*.

The “other” category of EOCs is comprised “different degradation products from unsaturated fatty acids, lactones, terpenes, glycosides, and sulfur- and nitrogen-containing compounds” Hyldgaard et al. *(30)*. This category includes allicin and ally isothiocyanate.

Variation of bacteria susceptibility to EOs and EOCs is likely due to variation in cell structure. Gram-positive bacteria are proposed to be more susceptible to EOs than gram-negative bacteria. Gram-positive bacteria are surrounded by a thick peptidoglycan wall. This layer provides structure to gram-positive bacteria, but small antimicrobial particles can still access the cell membrane. In comparison, gram-negative bacteria have an outer membrane composed of lipopolysaccharide (LPS) which creates a rigid, restrictive barrier against large molecular particles, especially hydrophobic compounds, including antimicrobials *(61)*. However, gram-negative bacteria are susceptible to EOCs. Helander et al. concluded cellular penetration of thymol and carvacrol on *E. coli*

and *S. Typhimurium* was the same. The transport of EOs into gram-negative bacteria is proposed to be due to the activity of porin proteins embedded in the outer membrane. Porin proteins transport hydrophilic and phenolic compounds to the cytoplasmic membrane and targets for antimicrobial activity *(29, 36)*.

A highly important characteristic that is believed to contribute to EOs and EOC antimicrobial activity is its amphiphilicity. The hydrophobic phenolic ring changes membrane functionality by partitioning in the lipids of the bacterial cell membrane. The accumulation of EOs influences the protein-to-lipid ratios in the membrane increasing permeability. However, due to the hydrophobicity of the phenolic ring, solubility in the lipids may inhibit the compound to act on the microorganism *(10, 14, 82)*. The hydrophilic phenolic hydroxyl group contributes to the antimicrobial activity by exchanging the hydroxyl proton for another ion to diffuse through the cytoplasmic membrane to the cytoplasm. This may lead to disruption of enzymatic activity *(10, 14, 95)*.

Combining natural antimicrobials to increase antimicrobial activity

While natural antimicrobials are active against a range of microorganisms, some have a limited spectrum. For example, an antimicrobial may be active against gram-positive bacteria and molds but not gram-negative bacteria. The difference in antimicrobial spectra is most likely due to variation in the target sites of the antimicrobial on microbial cells *(97)*. Combination of antimicrobials aims to

utilize differing antimicrobial mechanisms thereby targeting multiple sites and thus completely inhibiting a microorganism or microorganisms *(93)*.

Three different interactions can occur when combining antimicrobials: synergistic, additive, or antagonistic. The combination displays an additive effect when the antimicrobial blend results in an antimicrobial activity that is equal to the sum of the individual compounds. An antimicrobial blend resulting in a combined antimicrobial activity less than individual compounds applied separately is antagonism. A synergistic interaction occurs when a blend of two or more antimicrobial compounds results in an antimicrobial activity greater than the sum of the individual compounds. *(19)*.

Analysis of the combined effects of the antimicrobials can be done by calculating the fractional inhibitory concentration index (FIC_{index}). Davidson and Parrish defined the formula as: $FIC_A=MIC_{A+B}/MIC_A$, $FIC_A =MIC_{B+A}/MIC_B$, $FIC_{index} =FIC_A +FIC_B$ *(19)*. Thus, the MIC of compound A and B must first be determined for the individual components before calculating the FIC_{index}. The calculated values can then be interpreted as synergistic, additive, or antagonistic. In this thesis, we define a synergistic interaction as FIC_{index} *(6, 72)*.

Assessment of antimicrobial interactions can be performed in macro- or micro-dilution techniques in culture broth or agar media. Checkerboard, graphical, and time-kill assays are the most widely used methods to determine antimicrobial effects. However, there is no standard method to compare antimicrobial interactions. FIC values for the definition of interactions, methods,

and criteria used differ between studies. Thus it is often hard to compare antimicrobial interaction studies due to the diversity of methods *(6)*.

The purpose of studies combining plant extracts for synergy is to attempt to reduce use concentrations of antimicrobials and thus reduce sensory side effects, particularly for EOs and EOCs. Furthermore, combinations of antimicrobials may inhibit a broader spectrum of activity against microorganisms. Gutierrez et al. *(32)* conducted a study to determine the antimicrobial activity of binary plant essential oil combinations against *B. cereus*, *E. coli*, *L. monocytogenes*, and *P. aeruginosa*. Individual EO MICs for essential oils of basil, lemon balm, marjoram, oregano, rosemary, sage, and thyme were determined by a spot-on-agar test on tryptic soy agar for 24 h at 37°C. A checkerboard method was performed using a 96-well microtiter plate for binary combinations. The plate was arranged where EO_1 was serially diluted two-fold starting at its MIC along the x-axis of the plate and EO_2 was serially diluted two-fold starting at its MIC along the y-axis. The antimicrobials were combined with broth containing the microorganism strain and plates were incubated at 37°C for 24 h. FIC values were calculated to determine if the combinations resulted in a synergistic, antagonistic, or additive interaction. No growth at FIC ≤ 0.5 was defined as a synergistic interaction. No synergistic interactions were detected between the EO combinations studied by Gutierrez et al. *(32)*. This may be attributed to the means of EO selection based upon reported antimicrobial efficiency, sensory properties, and presence of different compounds. Synergy is

thought to occur when antimicrobial blends inhibit multiple enzymes or biochemical pathways or interact to disrupt the cell membrane structure or function *(6)*. Therefore, antimicrobial combination studies should look to combine antimicrobials with different proposed mechanisms for synergy.

Combination studies that utilize other natural plant-based antimicrobials could increase the antimicrobial activity of EOs and further decrease negative sensory side effects. Miyague et al. *(57)* evaluated the antimicrobial activity of phenolic acids and EOCs alone and in combination against *L. monocytogenes* in a micro-dilution broth checkerboard analysis. Combinations of carvacrol+o-coumaric, carvacrol+FA, carvacrol+p-hydroxybenzoic inhibited the growth of *L. monocytogenes* and had a synergistic interaction (FIC ≤ 0.5) at pH 5.

To further reduce use concentrations, increase the potential spectrum of microorganism inhibition, and reduce cost and sensory effects, combination studies between 3 or more antimicrobials could be conducted. Techathuvanan et al. *(93)* tested binary and tertiary antimicrobial combinations against pathogenic and spoilage microorganisms in a macro-dilution broth assay at 25°C and pH 6.0 to simulate ambient storage conditions and intrinsic pH of low acid food products. FICs were calculated for each study to define synergistic, additive, and antagonistic effects. A synergistic effect was found for the combination of white mustard essential oil + lauric arginate + citrus flavonoid and acid blend. In this study, original use concentrations (i.e., MICs) were reduced by 83.4% *(93)*.

Oregano

Oregano (*Origanum vulgare*) is a commonly used spice in the Mediterranean Basin, Philippines, and Latin American cuisines. The flowered tops and stalks can be dried and used as an herb. Distillation of dried oregano can extract oregano essential oil (OEO) *(44)*. Carvacrol, a monoterpenoid phenol, thymol (5-methyl-2-(1-methyl)-phenol), a monoterpenoid phenol, γ-terpinene (4-methyl-1-(1-methylethyl)-1,4-cyclohexadiene), a monoterpene, and ρ-cymene (1-Methyl-4-(1-methylethyl)benzene), a monoterpene, are the primary components of OEO *(10)*.

OEO generally has greater antibacterial activity in comparison to most other EOs *(10)*. It has been shown to inhibit both gram-negative and gram-positive bacteria as well as fungi *(17)*. Gutierrez et al. *(32)* reported that OEO had antimicrobial activity against *Bacillus cereus*, *E. coli*, *L. monocytogenes*, and *Pseudomonas aeruginosa*. Minimum inhibitory concentrations determined by a microbroth dilution assay in a 96-well micro titer plate at 37°C for 18 h in TSB were 500, 400, 200, and 200,000 mg/l, respectively. OEO has also shown to be effective in reducing biofilm growth of microorganisms. The biofilm growth of *S. aureus* was decreased in the presence of 0.0125% OEO after incubation for 24 h at 37°C. Direct observation by electron microscopy of *S. aureus* with 0.0125% OEO showed cells grew as loose colonies compared to the biofilm matrix of *S. aureus* in the absence of OEO *(62).*

The application of OEO to aid in food preservation and safety has been widely studied. Concentrations ranging from 0.05 to 1% of OEO in combination with modified atmosphere packaging were able to inhibit the growth of *Brochothrix thermosphacta* in minced meat stored at 5°C. Additionally, sensory analysis of the minced meat with 1% OEO concluded that OEO positively affected the odor and color of minced meat. OEO flavor was not detected by panelists *(85)*. Similarly, OEO at 0.8% in combination with modified packaging conditions had a 2-3 log reduction of *L. monocytogenes* on meat fillets at 5°C *(94)*. Cod and salmon fillets with 0.05% OEO in modified atmosphere packaging stored at 2°C inhibited the growth of the spoilage microorganism *Photobacterium phosphoreum (55)*.

The mode of action of oregano is proposed to be based on its major components, thymol and carvacrol. Ultee et al. *(96)* studied the effect of carvacrol on the intracellular ATP pool (ATP_{in}), the membrane potential, the pH-gradient across the cytoplasmic membrane, and the potassium gradient of gram-positive *B. cereus.* The study showed a decrease in ATP_{in} concentration but no increase in the extracellular ATP (ATP_{out}) concentration, a decrease of membrane potential by a change in pH from pH 7.0 to pH 5.8, and an increased permeability of the cell membrane for K^+ when treated with carvacrol. Thus it was concluded that “the hydrophobic carvacrol interacts with the membrane of *B. cereus* by changing permeability for cations such as H^+ and K^+“ *(96)*. In contrast, a study conducted by Helander et al. *(36)* observed an increase in ATP_{out} and ATP_{in} of

the gram-negative bacteria *E.coli* when exposed to carvacrol. The study also exposed *E.coli* to thymol, an EOC of OEO, to determine its mechanism of action. A measurement of lipopolysaccharide (LPS) release after exposure to carvacrol or thymol concluded that the EOCs potentially degraded the outer membrane of *E. coli*. Thus, the mode of action of carvacrol and thymol against gram-negative bacteria was proposed to be increased cell membrane permeability to ATP via degradation of the outer membrane and release of LPS *(36)*. Another suggested mode of antimicrobial action for carvacrol is related to the inhibition of motility of bacterial cells. Alphen et al. *(2)* treated *Campylobacter jejuni* with a sub-inhibitory concentration of carvacrol investigate the effect on influence virulence traits of *C. Jejuni* but not inhibit cellular growth. Time-lapse microscopy showed that *C. jejuni* flagella biosynthesis was not altered but the organism became non-motile in the presence of 0.2 mM (30 ppm) carvacrol. Additionally, a luciferase assay was used to determine if carvacrol had an effect on *C. jejuni* ATP levels. The assay showed similar ATP levels for *C. jejuni* grown with or without carvacrol. Therefore, Alphen et al. *(2)* concluded that carvacrol inhibited the motility of *C. jejuni* independent of ATP levels.

Coriander

Coriander (*Coriandrum sativum L.)* is an herb and spice native to the Mediterranean and Middle East. The leaves are more commonly referred to as cilantro while the seeds are called coriander. Coriander essential oil (CEO) is derived from the seeds *(80)*. Linalool (65-90%) a monoterpenoid alcohol, and α-

pinene (2,6,6-trimethylbicyclo[3.1.1]hept-2-ene) (5-90%), a monoterpene, are the primary antimicrobial components of CEO *(22)*.

CEO is reported to have antimicrobial activity against bacteria and yeasts *(17).* A study conducted by Delaquis et al. *(22)* found CEO (≤ 0.5 % v/v) in a microbroth dilution assay in TSBYE had antimicrobial activity against *Listeria monocytogenes*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, *Pseudomonas fragi*, and *Salmonella* Typhimurium at 30°C at 48 h. CEO (≤ 0.5 % v/v) extracted by hydrodistillation from dried fruits of *C. sativum* composed of 64.5% linalool and 6.3% α-pinene inhibited the growth of *Escherichia coli, Bacillus megaterium, and Erwinia carotovora* by a disk diffusion assay at 25°C for 48h *(50)*.

CEO has also been shown to have considerable antimicrobial activity in food matrix studies. Stecchini et al. applied 1250 µg/ml CEO to noncured cooked pork inoculated with *Aeromonas hydrophila*. The samples were stored at 2 and 10°C under vacuum or air packaging. The addition of CEO reduced the growth of *A. hydrophila* by 5-logs when stored under vacuum packaging at 2 and 10°C *(88)*. CEO (0.5% v/w) homogenized with lean beef and chicken breast inoculated with 5 log CFU/ml of *Campylobacter jejuni* caused a reduction in cell counts to an undetectable level after 30 min when stored at 4°C and 32°C *(73)*.

The mechanism of antimicrobial activity of CEO has been attributed to membrane damage of gram-negative and gram-positive bacteria. Silva et al. concluded from a flow cytometry study that CEO caused membrane damage

leading to cellular death. Cellular function was evaluated using several fluorochromes: propidium iodide (PI) for membrane integrity, bis-1,3-dibutylbarbutiric acid (BOX) for membrane potential, ethidium bromide (EB) for efflux activity, and 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) for respiratory activity. Cell suspensions of *B. cereus*, *S. aureus*, *E. coli*, *S. typhimurium*, and *P. aeruginosa* exposed to their CEO MIC lost all cellular functions including efflux and respiratory activity. This was shown by evaluating the percentage of fluorochrome-stained cells after treatment with CEO and comparing to a 2% DMSO control. Gram-negative bacteria, *E. coli* (MIC 0.2%), *P. aeruginosa* (MIC 1.6%), and *S. typhimurium* (MIC 0.4%), exhibited a higher susceptibility to CEO than gram-positive bacteria, *B. cereus* (MIC 0.1%) and *S. aureus* (MIC 0.1%). A second mode of antimicrobial action proposed for CEO is a chelating activity. Ahlers et al. *(1)* compared CEO ferrous ion chelating activity to ethylenediaminetetraacetic acid (EDTA), a compound known for its high chelating activity, using a chelation activity test. The MIC of CEO (1.0%) demonstrated a high chelation activity as its ability to bind ferrous ions resulted in 71.12 ± 0.48 % inhibition of the ferrozine-iron (II) complex. In comparison, 1% EDTA 94.16 ± 0.12% inhibition of the ferrozine-iron (II) complex.

Basil

Basil (*Ocimum basilicum*) is a glabrous (smooth) herb which prefers a warm and temperate climate for growth. Cultivation originated in India and tropical Asia, but it is now cultivated commercially in several European countries,

including France, Greece and Egypt and multiple areas of the United States *(69, 84)*. The EO of basil is produced by both the flower and herb. Composition of basil essential oil (BEO) has great variation depending on variety, geographic location, and time of harvest. Major components reported include linalool (35%-60%), geraniol (35-45%) a monoterpenoid alcohol, eugenol (20-25%) a phenylpropene, methyl chavicol (38%-50%), a phenylpropene, and camphor (20%), a terpenoid. BEO has a clove-like scent which is utilized as a flavoring agent in foods and scent in perfumes *(69)*.

Basil has shown inhibitory effects against both bacteria and fungi, including *Bacillus*, *Escherichia coli*, *Staphylococcus*, and *Aspergillus (17)*. In a study conducted by Bagamboula et al. *(3)*, BEO was extracted from dried basil by steam distillation. BEO (10% v/v) inhibited the growth of *Shigella flexneri*, *S. sonnei*, and *E.coli* in an agar well diffusion assay on Mueller Hinton Agar at 37°C for 24 h. In this study, BEO was shown to be composed of 16.1% linalool and no trace of eugenol. Additionally, antimicrobial activity of seasonal variations of BEO was compared in a study by Hussain et al. *(40)*. Stems and leaves were collected from *O. basilicum* L during summer (June), autumn (September), winter (December), and spring (March) in Faisalabad, Pakistan to compare concentration of EOC during varying temperatures and humidity. All seasonal variations of BEO had a linalool composition of approximately 60%. The four seasonal variations of BEO inhibited the growth of *S. aureus* (MIC 1.3 mg/ml), *E. coli* (MIC 2.6 mg/ml), *B. subtilis* (MIC 1.4 mg.ml), *P. multocida* (MIC 1.9 mg/ml),

A. niger (MIC 3.2 mg/ml), *M. mucedo* (MIC 4.9 mg/ml), and *F. solani* (MIC 3.6 mg/ml) in a microbroth dilution assay at 37°C for 24 h for bacteria and 30°C for 48 h for fungi *(40)*.

The internal and external addition of BEO to food products may aid in food preservation. Maize kernels coated with BEO (5.0%) with hexane as the solvent inhibited growth of *Aspergillus flavus.* The maize kernels were immersed in the BEO and hexane solution for 30 minutes then dried for an additional 30 minutes. The kernels were then sprayed *with A. flavus* spore suspension and incubated in petri dishes in wet cotton for 5 days at room temperature. The BEO maize kernel coating reduced A. *flavus* growth to 0.8% of 120 maize kernels after 5 days *(58, 90)*. BEO in combination with olive oil increased the death rate of *S. Enteritidis* in mayonnaise. Homemade mayonnaise (300 ml oil, 2 egg yolks, and 9 ml acidulate) with a pH of 4.3 was inoculated with *S.* Enteritidis and stored at 4°C and 20°C. Mayonnaise containing a proprietary brand of olive oil containing BEO (no reported concentration) had a 3 day death rate of *S.* Enteritidis as compared to sunflower oil with a 6 day death rate *(51)*.

BEO is proposed to destroy the integrity of a cell membrane resulting in cellular death. Lv et al. *(52)* studied the effect of BEO (MIC and 2xMIC) on *S. aureus*, *B. subtilis*, and *E. coli* by a scanning electron microscopy (SEM) microscopy test to see external damage of the cells after 2 hrs. The SEM test demonstrated that BEO at its MIC visually disrupted the membrane integrity of *S. aureus*, *B. subtilis*, and *E. coli*. BEO antimicrobial activity can also be attributed to

its high concentration of eugenol (0.25%-0.50%). Eugenol is proposed to cause cell lysis by leakage of proteins and lipids of both gram-negative and gram-positive bacteria. Oyedemi et al. exposed *L. monocytogenes* and *E. coli* to the MIC and 2 x MIC of eugenol in nutrient rich broth for 120 minutes at 37°C. At 30 minute intervals, the cell suspension was vortexed at 10,000 rpm to allow for leakage of cell constitutes. To measure the lipid leakage, a vanillin-phosphoric acid reagent which darkens in color in the presence of lipids was added to the cell suspension. Coomassie brilliant blue G-250 was added to the solution to stain proteins. Results showed that *L. monocytogenes* and *E.coli* treated with carvacrol (MIC 0.50%) had an increase of lipid and protein leakage compared to untreated bacterial cells by a darkening of color in the cell suspensions (absorbance measured at 595 nm) *(65)*.

Applications of EOs to foods as antimicrobials

It has been shown that EOs exhibit a stronger antimicrobial activity in microbiological media than in foods *(81)*. Application of EOs as antimicrobials in food systems is limited by interaction with proteins and lipid matrices. Vigil et al. attributed this to the interaction of amphiphilic compounds of EOs with hydrophobic proteins and lipids *(97)*. For example, EOs applied to the surface of meat may not treat the microbially contaminated center of the meat. Due to EOs being hydrophobic, the oils accumulate in the lipids on the meat surface *(29, 70)*. Other factors affecting the interaction of EOs is the neutral pH and high water activity of many foods *(87)*. These conditions are optimum for microorganisms

and often require an increased use concentration of EOs needed for antimicrobial activity. High concentrations of EOs can have negative sensory effects such as a change in aroma or taste. Thus solutions to overcome the weakened antimicrobial activity and sensory effects of EOs and their major components in food systems are needed in the food industry *(17)*.

Essentially the addition of antimicrobials to a food product is a hurdle that aids in the extension of shelf life and inhibition of food-borne pathogens. Other hurdles can be implemented during food processing to enhance antimicrobial activity. The combination of heat and a natural antimicrobial may have the ability to decrease both the concentration of antimicrobial and amount of heat required for processing. Non-thermal processing such as high hydrostatic pressure or pulsed electric fields with antimicrobials have also successfully aided in food preservation *(17)*. The combination of high-intensity pulsed electric field (HIPEF) with citric acid (2.0%) or cinnamon bark oil (0.20%) reduced the growth of *S.* Enteritidis, *L. monocytogenes*, and *E. coli* by more than 5.0 log CFU/ml in melon and watermelon juices. However, the taste and odor of the HIPEF juices did not pass sensory analysis *(60)*.

CHAPTER III

MATERIALS AND METHODS

Antimicrobials

The natural antimicrobials selected for this study were oregano essential oil (≥98% purity; Sigma-Aldrich, St. Louis, MO), coriander essential oil (≤100% purity; Sigma-Aldrich, St. Louis, MO), basil essential oil (≥98% purity; Sigma-Aldrich, St. Louis, MO), and ferulic acid (≤100% purity; MP Biomedicals, Solon, OH). A stock solution for each antimicrobial was prepared in dimethyl sulfoxide (DMSO; Fisher Scientific, Fair Lawn, NJ) immediately prior to the experiment. The antimicrobial stock solutions were diluted in tryptic soy broth with 0.6% yeast extract (TSBYE, pH 6.0; Difco, BD, Sparks, MD) for further experiments.

Bacteria

Listeria monocytogenes Scott A was obtained from the culture collection of the Department of Food Science and Technology at the University of Tennessee in Knoxville. *L. monocytogenes* was maintained at -80°C in 20% glycerol and grown in tryptic soy broth with 0.6% yeast extract (TSBYE, pH 6.0; Difco, BD, Sparks, MD) at 32°C for 24 h. The culture was transferred at least twice at 24 h intervals prior to use. *L. monocytogenes* grown overnight was serially diluted in 0.1% peptone (Difco, BD) and plated on TSAYE. Plates were incubated at 32°C for 48 h and colonies were counted.

Determination of minimum inhibitory concentrations (MICs)

A broth dilution assay was used to determine the MICs for the individual antimicrobials. A mixture of 0.1 ml antimicrobial stock solution in DMSO and 9.9 ml of TSBYE (pH 6.0) containing *L. monocytogenes* (10^4 CFU/ml) was incubated at 25°C for 48 h. Samples were serially diluted in a 0.1% peptone solution and spread onto TSAYE plates at time 0 and 48h. Colonies were counted after 48 h of incubation at 32°C. Two samples were taken per antimicrobial treatment per rep. All of the experiments were repeated at least twice. A negative control of DMSO without antimicrobial addition was also sampled. The MIC was defined as the lowest concentration resulting in a ≥ 1.0-log reduction in the bacterial test population.

Determination of combined antimicrobial effects

The influence of varying antimicrobial concentrations in binary, tertiary, and quaternary combinations was assessed against *L. monocytogenes* by a broth dilution assay. The design of the combinations used is shown in Table 1 and was modified from Techathuvanan et al. (*80*). All of the experiments were repeated at least twice. A combination of 0.1 ml aliquots of each antimicrobial solution was mixed with 9.7 ml (binary combination), 9.6 ml (tertiary combination), or 9.5 ml (quaternary combination) of TSBYE (pH 6.0). After vortexing antimicrobial combinations in TSBYE, 0.1 ml containing *L. monocytogenes* in TSBYE (final concentration10^4 CFU/ml) was added to the

solution *(93)*. Negative controls contained antimicrobial and sterile broth. Positive controls contained TSBYE inoculated with *L. monocytogenes* (10^4 CFU/ml) without antimicrobial. Antimicrobial controls were broth containing *L. monocytogenes* (10^4 CFU/ml) and a single antimicrobial at its MIC. Samples were incubated at 25°C for 48 h. Samples were serially diluted at 0 and 48 h in 0.1% peptone solution and spread onto TSAYE plates. Colonies were counted after 48 h incubation at 25°C.

FIC calculation

The fractional inhibitory concentrations (FICs) were calculated with the MIC of each antimicrobial by equations 1, 2, 3, and 4 *(19)*:

$$FIC_A = \frac{MIC\ of\ A\ in\ the\ presence\ of\ B,C,and/or\ D}{MIC\ of\ A\ alone} \quad (1)$$

$$FIC_B = \frac{MIC\ of\ B\ in\ the\ presence\ of\ A,C,and/or\ D}{MIC\ of\ B\ alone} \quad (2)$$

$$FIC_C = \frac{MIC\ of\ B\ in\ the\ presence\ of\ A,B,and/or\ D}{MIC\ of\ C\ alone} \quad (3)$$

$$FIC_D = \frac{MIC\ of\ B\ in\ the\ presence\ of\ A,B,and/or\ C}{MIC\ of\ D\ alone} \quad (4)$$

Once FICs were calculated, the fractional inhibitory concentration index (FIC_{index}) for tested antimicrobials was determined by equation 5, 6, and 7:

$$\text{Binary } FIC_{index} = FIC_A + FIC_B \quad (5)$$

$$\text{Tertiary } FIC_{index} = FIC_A + FIC_B + FIC_C \quad (6)$$

$$\text{Quaternary } FIC_{index} = FIC_A + FIC_B + FIC_C + FIC_D \quad (7)$$

The resulting FIC_{index} value represents the effects of the antimicrobial blend. The synergistic, additive, and antagonistic effects of antimicrobials was defined as an FIC_{index} ≤0.5, >0.5-1, and >1.5, respectively *(6, 72)*.

CHAPTER IV

RESULTS AND DISCUSSION

MICs of single antimicrobials

A study of MICs of EOs and a hydroxycinnamic acid was conducted at 25°C and pH 6.0, to simulate ambient storage conditions and intrinsic pH of low acid food products, using a broth dilution assay *(93)*. The MICs, defined as a ≥1.0-log reduction after incubation for 48 h, for *L. monocytogenes* Scott A were 250 ppm for oregano EO, 2,500 ppm for coriander EO, 7,500 ppm for basil EO, and 5,000 ppm for ferulic acid (Table 2). For OEO and CEO, the lethality increased significantly above the defined MICs while for BEO and FA, little increase in lethality was found for concentrations above the defined MICs. Thus, inhibition/inactivation by OEO and CEO could have been via a different mechanism than BEO and FA.

The MICs obtained in the present study are somewhat difficult to compare to previous studies because of the definition used. In most studies using microbroth dilution assays, inhibition is determined by monitoring growth spectrophotometrically or visually *(33, 75)*. In these types of studies, it is impossible to know if lack of growth is due to inhibition (stasis) or inactivation (cidal). In the present study, the actual count for a treatment was done at the endpoint and the MIC was a measure of lethality. A quantitative one log reduction from the initial count yields a more precise measurement of reduction in viable

cells in comparison to optical density measurements and visual evaluation of turbidity which cannot detect log reduction. While the methods are different, the MICs determined in the present study were similar to previous studies. For example, Gutierrez et al. *(32)* and Oussalah et al. *(64)* reported OEO MICs of 250 to 300 ppm against *L. monocytogenes* using an agar dilution assay in which the MIC was defined as the concentration of essential oil that completely inhibited visual growth of the test microorganism on the surface of an agar plate after 48 h at 32°C. Gutierrez et al. *(32)* also found an MIC for BEO of 10,000 ppm for *L. monocytogenes*. Delaquis et al. *(21)* and Oussalah et al. *(64)* reported an MIC for CEO of 8,000 - 10,000 ppm against *L. monocytogenes*. Delaquis et al. *(21)* determined the MIC of CEO by an agar dilution assay in a 96 well microtiter plate at 30°C for 48 h. MICs were the lowest antimicrobial concentration that resulted in complete inhibition of *L. monocytogenes* as determined visually. Varying values for the MIC of FA ranging from 1,500 to 10,000 ppm against *L. monocytogenes (8, 56, 91).* The wide range of MIC values may be due to the pH used in the studies however pH was not reported in the studies. Ferulic acid (pK_a = 4.42) has been shown to have the greatest antimicrobial activity when the acid is more undissociated at pHs between 4.5-4.9 *(11, 98)*. At higher pHs, FA dissociates making it more polar and decreasing its ability to cross the hydrophobic cell membrane to affect cellular metabolic activity. Miyague et al. *(57)* demonstrated the effect of pH on the activity of FA in a microbroth dilution assay using *L. monocytogenes*. The MIC of FA at pH 6.0 was 5000 ppm while at

pH 5.0 it was 2500 ppm after incubation for 48 h at 30°C. In the study, the MIC was defined as the lowest concentration where no increase in optical density at 600 nm occurred.

Efficacy of antimicrobial combinations

Spice essential oils are used for flavoring agents in foods and thus they contribute strong tastes and aromas. If EOs were to be used as antimicrobial agents, they would likely need to be applied at concentrations greater than that used for flavoring. Thus the sensory contributions by OEO, BEO, and CEO would be a major limitation to their use in foods. For example, OEO is described as having a green, fruity aroma, BEO an earthy, green aroma, and CEO a fruity and sweet, rose-like aroma *(74, 84, 85)*. When applied to food items, these sensory qualities may be interpreted as off flavors or scents attributed to spoilage *(85)*. Strategies for overcoming negative sensory contributions by EOs have included application to compatible foods (e.g., OEO in spaghetti sauce), addition through packaging or in gaseous forms, and encapsulation in various matrices. A more simple solution might be to reduce the concentration of individual antimicrobials by using combinations of EOs. Therefore, the ultimate goal of this project was to potentially reduce the concentration of EOs necessary for microbial inhibition and at the same time reduce sensory impact of the EOs in foods. The antimicrobial activity of OEO, CEO, BEO, and FA combinations was evaluated using a broth dilution assay. The antimicrobial activity of the combinations tested was

determined using a modified “checkerboard” assay and FIC values *(93)*. Table 1 shows the combinations tested and FIC_{index} of antimicrobial combinations.

Checkerboard, graphical, and time-kill assays using macro- or microdilution techniques are commonly used to study the effects of antimicrobial combinations on microorganisms *(6)*. To determine synergistic, additive, or antagonistic interactions, FIC values are calculated. However, there is currently no standard method to define FIC values. Therefore, comparison of studies on antimicrobial effects can be challenging *(6, 93)*. In the present study, the antimicrobial combination design of Techathuvanan et al. *(93)* was modified for combinations of four antimicrobials (Table 1). An MIC for combinations was defined as the concentrations causing <1 log CFU/ml growth. Previously, antimicrobial combination studies using FICs have generally only reported growth vs. no growth of the test microorganism *(32, 57, 64, 102)*. Using log reductions of *L. monocytogenes* allows for more quantitative comparisons between the antimicrobial combinations.

Binary antimicrobial combinations

The efficacy of binary combinations of OEO+CEO, OEO+BEO, OEO+FA, CEO+BEO, CEO+FA, and BEO+FA at pH 6.0 and 25°C is shown in Table 3. The calculated FIC values are displayed on the farthest left side of the table. As with the antimicrobial tests to determine individual MICs, an MIC for combinations was defined as the concentrations causing at least a one log CFU/ml reduction.

A synergistic interaction occurred when FIC≤0.5 had a one log CFU/ml reduction. An additive interaction occurred when an FIC<1.5 had a one log CFU/ml reduction. An antagonistic interaction occurred when FIC>1.5, FIC=1, and a FIC≤0.5 had less than a one log CFU/ml reduction after 48 h incubation at 25°C. The fractional proportion of the MIC used for each antimicrobial is reported in second ("A") and third columns ("B") for the antimicrobial combinations. Antimicrobial A is the first antimicrobial listed in the combination while antimicrobial B is the second antimicrobial listed in the combination. The concentration of antimicrobial in the combination can be calculated by multiplying the MIC fractional proportion by the MIC of each individual antimicrobial (OEO = 250 ppm, CEO = 2500 ppm, BEO = 7500 ppm, FA = 5000 ppm). Thus for OEO+BEO at an FIC of 1 and ½ of A and ½ of B, the concentration of OEO would be 125 ppm and of BEO 3750 ppm.

The lowest FIC_{Index} = 0.5 indicated synergistic antimicrobial activity against *L. monocytogenes* and utilized a combination of ¼ antimicrobial MIC and ¼ antimicrobial MIC proportions of OEO+BEO, OEO+CEO, CEO+BEO, CEO+FA, and BEO+FA (FIC_{index} 0.5). The combinations of OEO+BEO, OEO+CEO, CEO+BEO, CEO+FA and BEO+FA had a > 1.0 log reduction at an FIC = 0.5 and were therefore synergistic. Binary combinations with a synergistic effect reduced use concentrations of single antimicrobials by 75%. When OEO+FA were applied against *L. monocytogenes*, an additive effect was obtained (FIC_{index}=1) (Table 3). This was shown by a less than 1 log CFU/ml reduction at an FIC of 0.5 but a

greater than 1 log CFU/ml reduction at one of the combinations of FIC = 1, i.e., ¼ OEO and ¾ FA.

The synergistic combinations of OEO+BEO, OEO+CEO, CEO+BEO, and BEO+FA at an FIC at 0.5 resulted in a <1 log CFU/ml growth of *L. monocytogenes* at pH 6.0 and 25°C. In contrast, the synergistic combination of CEO+FA resulted in a 1 log CFU/ml reduction (3.08±0.08 log CFU/ml growth after 48 h incubation at 25°C) of *L. monocytogenes* (Table 3). The greater inhibitory effect of the combinations of OEO+BEO, OEO+CEO, CEO+BEO, and BEO+FA may be attributed to the mechanisms of antimicrobial action being more complimentary enhancing antimicrobial activity.

Gutierrez et al. *(32)* reported an indifference (defined as a combination of antimicrobials that results in an absence of interaction *(6)*) when treating *L. monocytogenes* with 10,000 ppm basil and 100 ppm oregano in a microdilution broth assay at 37°C for 18 h. However, an additive effect was reported against *B. cereus*, *E. coli*, and *P. aeruginosa (32)*. Bassole et al. *(6)* reported a synergistic antimicrobial effect against *L. monocytogenes* by combining carvacrol with linalool in a microdilution broth assay. The major antimicrobial component of OEO is carvacrol which has also been shown to have high antimicrobial activity against microorganisms *(17)*. Linalool is a main component in CEO and BEO *(69, 80)*. Therefore, the synergistic effect of combining OEO+BEO and OEO+CEO could be attributed to interactions between its major components carvacrol and linalool (Table 3). Additionally, eugenol is also a component of

BEO. Pei et al. *(67)* found a synergistic effect between 100 ppm carvacrol and 800 ppm eugenol in a macrodilution broth assay at 37°C for 24 h against *E. coli* which can be compared to the synergistic interaction of OEO+BEO. Miyague et al. *(57)* studied the combination of FA and carvacrol against *L. monocytogenes* at pH 5.0 and pH 6.0. A synergistic interaction was reported at pH 5.0 while an indifference interaction was reported at pH 6.0 *(57)*.

Tertiary antimicrobial combinations

The efficacy of tertiary combinations of OEO+CEO+BEO, OEO+CEO+FA, OEO+BEO+FA, and CEO+BEO+FA at pH 6.0 and 25°C is shown in Table 4. The combination of OEO+CEO+BEO and CEO+BEO+FA, the combination of OEO+BEO+FA at a FIC at 1.5, and the combination of OEO+CEO+FA at a FIC≥1.0 resulted in inactivation of *L. monocytogenes* at pH 6.0 and 25°C.

A synergistic interaction occurred when FIC≤0.5 had a one log CFU/ml reduction. An additive interaction occurred when an FIC<1.5 had a one log CFU/ml reduction. An antagonistic interaction occurred when FIC>1.5, FIC=1, and a FIC≤0.5 had less than a one log CFU/ml reduction after 48 h incubation at 25°C. The fractional proportion of the MIC used for each antimicrobial is reported in second (“A”), third columns (“B”), and fourth columns (“C”) for the antimicrobial combinations. Antimicrobial A is the first antimicrobial listed in the combination, antimicrobial B is the second antimicrobial listed in the combination, and antimicrobial C is the third antimicrobial listed in the combination. The

antimicrobial concentration in the combination can be calculated by multiplying the MIC fractional proportion by the MIC of the antimicrobial. The calculated FIC values are displayed on the farthest left side of the table. A synergistic interaction occurred when FIC≤0.5 had a one log CFU/ml reduction. An additive interaction occurred when an FIC=1 had a one log CFU/ml reduction. Within antimicrobial combinations, different concentrations of the antimicrobials with the same FIC_{index} resulted in varying inhibitory effects on *L. monocytogenes.* This can be seen in the OEO+CEO+FA tertiary combination. At FIC=1, 1/3OEO+1/3CEO+1/3FA had a <1 log CFU/ml growth after 48 hours while 1/2OEO+1/4CEO+1/4CEO had a >1 log CFU/ml growth after 48 hours (Table 4).

An additive effect was found for OEO+CEO+FA (FIC=1) against *L. monocytogenes*. Combinations of OEO+CEO+BEO, BEO+CEO+FA, and OEO+BEO+FA resulted in a synergistic effect (FIC≤0.5) (Table 6). Synergistic combinations resulted in an 83.4% concentration decrease of the tested antimicrobials. This was calculated by dividing the final use concentration of antimicrobial A by its MIC then subtracting the percent obtained from 100%. For example, for CEO, the final use concentrations was (1/6)*(2500 ppm) = 416.6 ppm. Dividing 416.67/2500 ppm is 16.6%. Subtracting 100%-16.6%, the concentration decrease was 83.4% from the original concentration.

Quaternary antimicrobial combinations

The efficacy of quaternary combination of OEO+CEO+BEO+FA at pH 6.0 and 25°C is shown in Table 5. The combination of OEO+BEO+CEO+FA at a FIC at 0.5 resulted in inhibition of *L. monocytogenes* at pH 6.0 and 25°C. The fractional MIC proportion is reported in the first column for the antimicrobial combinations. The antimicrobial concentration in the combination can be calculated by multiplying the MIC fractional proportion by the MIC of the antimicrobial. The calculated FIC values are displayed on the farthest left side of the table. A synergistic interaction occurred when FIC≤0.5 had 1 log CFU/ml growth reduction. Similar to tertiary combinations, different concentrations of the antimicrobials with the same FIC_{index} resulted in varying inhibitory effects on *L. monocytogenes* (Table 5). The combination of OEO+CEO+BEO+FA resulted in a synergistic interaction (Table 6).

The three EOs and a HA chosen for this study were based on proposed antimicrobial mechanisms. The macrobroth dilution assay had an incubation temperature of 25°C. One important characteristic of *L. monocytogenes* is that it is able to produce peritrichous flagella on the cell surface only at 20-25°C *(66)*. A proposed mechanism of OEO is that it inhibits flagella motility of bacterial cells *(2)*. OEO may also disrupt the cellular membrane *(2, 10)*. CEO can chelate transitional metals and cause membrane damage *(83, 92)*. BEO is proposed to bind to lipids and proteins preventing enzyme activity and causing cellular leakage *(6, 40, 99)*. FA has been reported to inhibit ATPase activity of cells *(76)*.

Synergy is thought to occur when the antimicrobial blend inhibits several targets (e.g., biochemical pathways, enzymes, cell membrane) *(6)*. Therefore, the hypothesis was that combinations of these natural antimicrobials which act on different targets of bacteria would be good candidates for having synergistic antimicrobial interactions.

The mechanism of antimicrobial action of binary combinations of EOs has been the focus of several studies. Zhou et al. *(102)* hypothesized two possible synergistic interactions between cinnamaldehyde and carvacrol against *S.* Typhimurium. Carvacrol increases the permeability of the cytoplasmic membrane thus enabling cinnamaldehyde to be transported into the cell. The second hypothesis suggested cinnamaldehyde binds to proteins in the cell membrane creating pores and carvacrol increases the size of the pores to further disrupt the cellular membrane *(6)*. A study by Pei et al. *(67)* hypothesized a similar interaction for carvacrol and eugenol, the major components of OEO and BEO, respectively. They suggested that carvacrol disrupted the outer membrane of *E. coli* so that eugenol could enter the cytoplasm and its hydroxyl group could combine with proteins, preventing enzyme action.

Based on previous antimicrobial mechanism studies, several hypotheses could be put forth to explain the synergistic interactions. As previously mentioned, a synergistic interaction is proposed to occur when the antimicrobial blend inhibits several biochemical pathways, inhibits the activity of protective enzymes, or interacts with the cell wall or membrane *(6)*. Binary synergistic

interactions were OEO+BEO, OEO+CEO, CEO+BEO, CEO+FA, and BEO+FA. First, the synergy between OEO+BEO may be due to OEO disrupting the cytoplasmic membrane of *L. monocytogenes* allowing BEO to enter the cell and bind to proteins, preventing enzymatic activity *(67)*. Similarly, for the combination of CEO+BEO, CEO disrupts the cytoplasmic membrane of *L. monocytogenes* allowing BEO to enter the cell resulting in the same enzymatic inhibition. Synergy between BEO+FA and CEO may be explained by BEO or CEO disrupting the membrane of *L. monocytogenes* to enable FA to be more easily transported into the cell and inhibit ATPase activity. The combination of OEO+CEO had a synergistic interaction due to the EOs acting on different parts of or components of the cytoplasmic membrane of *L. monocytogenes*.

Tertiary synergistic interaction of OEO+CEO+BEO, OEO+BEO+FA, and BEO+CEO+FA is due to OEO and CEO interacting with and disrupting the cellular membrane of *L. monocytogenes*, making it easier for BEO to enter and bind to proteins, inactivating enzymatic activity of the cell. The disruption of the cell membrane also increases permeability of FA to inhibit ATPase activity.

It can be hypothesized that the synergistic combination of OEO+BEO+CEO+FA is due to each antimicrobial acting on different targets to inhibit the growth of *L. monocytogenes* together. CEO and OEO together interact with and disrupt the cellular membrane of *L. monocytogenes*. OEO increases the permeability of the membrane while CEO acts as a chelating agent binding to transition metals. This disintegration of the membrane makes it easier for FA and

BEO to enter the cell. FA can then inhibit the ATPase activity of the cell and BEO can bind to lipids and proteins thus preventing enzymatic action of the cell. Synergism between HAs and EOs could have a major impact on the use of EOs as natural antimicrobials in the food industry since the sensory impact of EOs is the main challenge for their use in food products *(10, 57)*. Quaternary antimicrobial combinations resulted in an 87.5% reduced use concentration. Additionally, even an additive effect of FA with EOs could still maintain product safety while reducing use concentrations. The complementary mechanisms of FA with BEO, CEO, and/or OEO increased antimicrobial effectiveness. In combination, the complimentary mechanisms of OEO, CEO, BEO, and FA may be useful for the control of *Listeria* in foods.

In summary, the antimicrobial activities of OEO, BEO, CEO, and FA were enhanced through combination. To our knowledge, this is the first known report of a quaternary combination of antimicrobials. The synergistic addition of OEO+BEO+CEO+FA decreased the concentration of each antimicrobial significantly. Further studies should be conducted to evaluate the application of these antimicrobial combinations in food models.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

The plant derived natural antimicrobials tested in this study were effective in inhibiting the growth of *Listeria* under *in vitro* conditions. The addition of these compounds to food products may improve food safety and increase shelf life while maintaining a “clean label.” The combination of antimicrobials with different proposed mechanisms of action may affect multiple biochemical processes of a microorganism. These interactions create hurdles that can inhibit the growth of or inactivate the microorganism *(6, 10, 93)*. As previously mentioned, both synergistic and additive effects lower the antimicrobial use concentration decreasing sensory impact while preserving microbial safety and food quality. Previous studies have shown that EO mixtures may be suitable to control growth of *L. monocytogenes* in ready-to-eat raw or cooked foods *(32)*. Therefore, EOs plus HAs may have potential to be used in foods to eliminate *L. monocytogenes.*

The results from the present study show that combinations of OEO, CEO, BEO, and FA reduced use concentrations and inhibited the growth of *L. monocytogenes*. Quaternary combination of antimicrobials with proposed different mechanisms act on different targets to inhibit the growth of a microorganism together. The quaternary combination of OEO+CEO+BEO+FA has potential to be used in foods to inactivate *L. monocytogenes* without causing significant sensory effects. Further studies should be conducted to evaluate the

efficiency of synergistic and additive combinations within a food system on *L. monocytogenes*.

LIST OF REFERENCES

1. Ahlers, A.S. 2015. Characterization of the antimicrobial activity of coriander essential oil against foodborne pathogenic bacteria. *In*, Universität Hohenheim Institut für Lebensmittelwissenschaft und Biotechnologie.
2. Alphen, L. B. v., S. A. Burt, A. K. J. Veenendaal, N. M. C. Bleumink-Pluym, and J. P. M. v. Putten. 2012. The Natural Antimicrobial Carvacrol Inhibits Campylobacter jejuni Motility and Infection of Epithelial Cells. *PLoS ONE*. 7:1-7.
3. Bagamboula, C. F., M. Uyttendaele, and J. Debevere. 2004. Inhibitory effect of thyme and basil essential oils, carvacrol, thymol, estragol, linalool and p-cymene towards *Shigella sonnei* and *S. flexneri. Food Microbiology*. 21:33-42.
4. Baird-Parker, T. 2000. The production of microbiologically safe and stable foods. *The microbiological safety and quality of food*. 1:3-18.
5. Barmpalia, I., I. Geornaras, K. Belk, J. Scanga, P. Kendall, G. Smith, and J. Sofos. 2004. Control of *Listeria monocytogenes* on frankfurters with antimicrobials in the formulation and by dipping in organic acid solutions. *Journal of Food Protection*. 67:2456-2464.
6. Bassole, I. H. N., and H. R. Juliani. 2012. Essential oils in combination and their antimicrobial properties. *Molecule*. 17:3989-4006.
7. Bell, C., and A. Kyriakides. 2012. *Listeria*: a practical approach to the organism and its control in foods. Springer Science & Business Media.
8. Borges, A., C. Ferreira, M. J. Saavedra, and M. Simões. 2013. Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. *Microbial Drug Resistance*. 19:256-265.
9. Branen, A. L., and R. J. Haggerty. 2002. Introduction to food additives. p. 1-9. *In* A.L. Branen, et al. (ed.), Food additives Marcel Dekker, New York.
10. Burt, S. 2004. Essential oils: their antibacterial properties and potential application in foods-a review. *International Journal of Food Microbiology*. 94:223-253.
11. Campos, F. M., J. A. Couto, A. R. Figueiredo, I. V. Tóth, A. O. S. S. Rangel, and T. A. Hogg. 2009. Cell membrane damage induced by phenolic acids on wine lactic acid bacteria. *International Journal of Food Microbiology*. 135:144-151.
12. Chung, K.T., and C. A. Murdock. 1991. Natural systems for preventing contamination and growth of microorganisms in foods. *Food structure*. 10:10.
13. Datta, A. R. 2003. Listeria monocytogenes. p. 105-121. *In* M.D. Miliotis, and J.W. Bier (ed.), International handbook of foodborne pathogens Marcel Dekker.
14. Davidson, P., and A. Branden. 1981. Antimicrobial activity of non-halogenated phenolic compounds. *Journal of Food Protection*. 44:623-632.
15. Davidson, P., and A. Branen. 2005. Food antimicrobials-an introduction. *Food Science And Technology-New York-Marcel Dekker*. 145:1.
16. Davidson, P. M. 1993. Parabens and Phenolic Compounds. p. 263-306. *In* P.M. Davidson, and A.L. Branen (ed.), Antimicrobials in Foods Marcel Dekker, Inc, New York, New York.

17. Davidson, P. M., F. J. Critzer, and T. M. Taylor. 2013. Naturally Occurring Antimicrobials for Minimally Processed Foods. *Annual Review of Food Science and Technology*. 4:163-190.
18. Davidson, P. M., and M. A. Harrison. 2002. Resistance and adaptation to food antimicrobials, sanitizers, and other process controls. *Food Technology-Champaign Then Chicago*. 56:69-78.
19. Davidson, P. M., and M. E. Parish. 1989. Methods for testing the efficacy of food antimicrobials. *Food Technology*. 43:148-155.
20. Davidson, P. M., T. M. Taylor, and S. E. Schmidt. 2013. Chemical Preservatives and Natural Antimicrobial Compounds. p. 765-801. *In* M.P. Doyle, and R.L. Buchanan (ed.), Food Microbiology:Fundamentals and Frontiers ASM Press, Washington, D.C. .
21. Delaquis, P. J., K. Stanich, B. Girard, and G. Mazza. 2002. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *International Journal of Food Microbiology*. 74:101-109.
22. Delaquis, P. J., K. Stanich, B. Girard, and G. Mazza. 2002. Antimicrobial activity of individual and mixed fractions of dill, cilantro, coriander and eucalyptus essential oils. *International Journal of Food Microbiology*. 74:101-109.
23. Denyer, S., and W. B. Hugo. 1991. Mechanisms of action of chemical biocides. Their study and exploitation. Blackwell Scientific Publications.
24. Donnelly, C. W., and E. H. Briggs. 1986. Psychrotrophic growth and thermal inactivation of *Listeria monocytogenes* as a function of milk composition. *Journal of Food Protection*. 49:994-1002.
25. Escarpa, A., and M. C. Gonzalez. 2010. An Overview of Analyrical Chemistry of Phenolic Compounds in Foods. *Critical Reviews in Analytical Chemistry*. 31:57-139.
26. Eskin, N. AM. 1990. Biochemistry of Foods. *In* Academic Press, San Diego, CA.
27. Farber, J., and P. Peterkin. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiological reviews*. 55:476.
28. Gahan, C., B. O'Driscoll, and C. Hill. 1996. Acid adaptation of *Listeria monocytogenes* can enhance survival in acidic foods and during milk fermentation. *Applied and Environmental Microbiology*. 62:3128-3132.
29. Gann, L. D. 2013. Antimicrobial activity of essential oil and their components against lactic acid bacteria. *In*, Master's Thesis University of Tennessee.
30. Gill, A., and R. Holley. 2006. Inhibition of membrane bound ATPases of *Escherichia coli* and *Listeria monocytogenes* by plant oil aromatics. *International Journal Of Food Microbiology*. 111:170-174.
31. Glass, K. A., D. A. Granberg, A. L. Smith, A. M. McNamara, M. Hardin, J. Mattias, K. Ladwig, and E. A. Johnson. 2002. Inhibition of *Listeria monocytogenes* by sodium diacetate and sodium lactate on wieners and cooked bratwurst. *Journal of Food Protection*. 65:116-123.

32. Gutierrez, J., C. Barry-Ryan, and P. Bourke. 2008. The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. *International Journal of Food Microbiology*. 124:91-97.
33. Hammer, K. A., C. Carson, and T. Riley. 1999. Antimicrobial activity of essential oils and other plant extracts. *Journal Of Applied Microbiology*. 86:985-990.
34. Harborne, J. B. 1989. Methods in plant biochemistry. Volume 1. Plant phenolics. Academic Press Ltd.
35. Harris, R. 2002. Synergism in the essential oil world. *International Journal of Aromatherapy*. 12:179-186.
36. Helander, I. M., H.-L. Alakomi, K. Latva-Kala, T. Mattila-Sandholm, I. Pol, E. J. Smid, L. G. M. Gorris, and A. v. Wright. 1998. Characterization of the Action of Selected Essential Oil Compoenets on Gram-negative Bacteria. *J. Agric. Food Chem*. 46:3590-3595.
37. Herald, P., and P. Davidson. 1983. Antibacterial activity of selected hydroxycinnamic acids. *Journal of Food Science*. 48:1378-1379.
38. Herrmann, K., and C. W. Nagel. 1989. Occurrence and content of hydroxycinnamic and hydroxybenzoic acid compounds in foods. *Critical Reviews in Food Science & Nutrition*. 28:315-347.
39. Ho, C.T. 1992. Phenolic compounds in food: an overview. *In*, ACS symposium series (USA).
40. Hussain, A. I., F. Anwar, S. T. Hussain Sherazi, and R. Przybylski. 2008. Chemical composition, antioxidant and antimicrobial activities of basil (*Ocimum basilicum*) essential oils depends on seasonal variations. *Food Chemistry*. 108:986-995.
41. Hyldgaard, M., T. Mygind, and R. L. Meyer. 2012. Essential oils in food preservation: mode of action, synergies, and interactions with food matrix components.
42. Information, N. C. f. B. Date, 2016, Ferulic Acid. Available at: https://pubchem.ncbi.nlm.nih.gov/compound/ferulic_acid#section=Top. Accessed May 5, 2016, 2016.
43. Knobloch, K., A. Pauli, B. Iberl, H. Weigand, and N. Weis. 1989. Antibacterial and antifungal properties of essential oil components. *Journal of Essential Oil Research*. 1:119-128.
44. Kulisic, T., A. Radonic, V. Katalinic, and M. Milos. 2004. Use of different methods for testing antioxidant activity of oregano essential oil. *Food Chemistry*. 85:633-640.
45. Lado, B. H., and A. E. Yousef. 2007. Characteristics of *Listeria monocytogenes* important to food processors. *Food Science And Technology-New York-Marcel Dekker*. 161:157.
46. Lambert, R. J. W., P. N. Skandamls, P. J. Coote, and G. J. E. Nychas. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol, and carvacrol. *Journal of Applied Microbiology*. 91:453-463.

47. Langenheim, J. H. 1994. Higher plant terpenoids: a phytocentric overview of their ecological roles. *Journal of chemical ecology*. 20:1223-1280.
48. Larson, A., E. Johnson, and J. Nelson. 1999. Survival of *Listeria monocytogenes* in commercial cheese brines. *Journal of Dairy Science*. 82:1860-1868.
49. Lianou, A., and J. N. Sofos. 2007. A review of the incidence and transmission of *Listeria monocytogenes* in ready-to-eat products in retail and food service environments. *Journal of Food Protection*. 70:2172-2198.
50. Lo Cantore, P., N. S. Iacobellis, A. De Marco, F. Capasso, and F. Senatore. 2004. Antibacterial activity of *Coriandrum sativum L*. and *Foeniculum vulgare Miller var. vulgare* (Miller) essential oils. *Journal of Agricultural And Food Chemistry*. 52:7862-7866.
51. Lock, J., and R. Board. 1995. The influence of acidulants and oils on autosterilization of home-made mayonnaise. *Food Research International*. 28:569-572.
52. Lv, F., H. Liang, Q. Yuan, and C. Li. 2011. In vitro antimicrobial effects and mechanism of action of selected plant essential oil combinations against four food-related microorganisms. *Food Research International*. 44:3057-3064.
53. Lyon, G., and F. McGill. 1988. Inhibition of growth of *Erwinia carotovora* in vitro by phenolics. *Potato Research*. 31:461-467.
54. Marwan, A. G., and C. W. Nagel. 1986. Microbial inhibitors of cranberries. *Journal of Food Science*. 51:1009-1013.
55. Mejlholm, O., and P. Dalgaard. 2002. Antimicrobial effect of essential oils on the seafood spoilage micro-organism *Photobacterium phosphoreum* in liquid media and fish products. *Letters in Applied Microbiology*. 34:27-31.
56. Merkl, R., I. Hradkova, V. Filip, and J. Smidrkal. 2010. Antimicrobial and antioxidant properties of phenolic acids alkyl esters. *Czech J Food Sci*. 28:275-279.
57. Miyague, L., R. E. F. Macedo, G. Meca, R. A. Holley, and F. B. Luciano. 2015. Combination of phenolic acids and essential oils against Listeria monocytogenes. *LWT - Food Science and Technology*. 64:333-336.
58. Montes-Belmont, R., and M. Carvajal. 1998. Control of *Aspergillus flavus* in maize with plant essential oils and their components. *Journal of Food Protection*. 61:616-619.
59. Morey, A., C. Bratcher, M. Singh, and S. McKee. 2012. Effect of liquid smoke as an ingredient in frankfurters on *Listeria monocytogenes* and quality attributes. *Poultry Science*. 91:2341-2350.
60. Mosqueda-Melgar, J., R. M. Raybaudi-Massilia, and O. Martín-Belloso. 2008. Combination of high-intensity pulsed electric fields with natural antimicrobials to inactivate pathogenic microorganisms and extend the shelf-life of melon and watermelon juices. *Food Microbiology*. 25:479-491.
61. Nikaido, H. 1994. Prevention of drug access to bacterial targets: permeability barriers and active efflux. *Science*. 264:382-388.

62. Nostro, A., A. S. Roccaro, G. Bisignano, A. Marino, M. A. Cannatelli, F. C. Pizzimenti, P. L. Cioni, F. Procopio, and A. R. Blanco. 2007. Effects of oregano, carvacrol and thymol on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms*. Journal of Medical Microbiology*. 56:519-523.
63. Ou, S., and K. C. Kwok. 2004. Ferulic acid: pharmaceutical functions, preparation and applications in foods*. Journal of the Science of Food and Agriculture*. 84:1261-1269.
64. Oussalah, M., S. Caillet, L. Saucier, and M. Lacroix. 2007. Inhibitory effects of selected plant essential oils on the growth of four pathogenic bacteria: *E. coli O157:H7, Salmonella Typhimurium, Staphylococcus aureus* and *Listeria monocytogenes. Food Control*. 18:414-420.
65. Oyedemi, S. O., A. I. Okoh, L. V. Mabinya, and G. Pirochenva. 2009. The proposed mechanism of bactericidal action of eugenol, a-terpineol, and y-terpinene against *Listeria monocytogenes, Streptococcus pyogenes, Lactobaccilus vulgaris*, and *Escherichia coli. African journal of biotechnology*. 8:1290-1286.
66. Peel, M., W. Donachie, and A. Shaw. 1988. Temperature-dependent expression of flagella of *Listeria monocytogenes* studied by electron microscopy, SDS-PAGE and Western blotting*. Journal of General Microbiology*. 134:2171-2178.
67. Pei, R. s., F. Zhou, B. p. Ji, and J. Xu. 2009. Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol, and carvacrol against *E. coli* with an improved method*. Journal of Food Science*. 74:M379-M383.
68. CDC. 2015, Multistate Outbreak of Listeriosis Linked to Blue Bell Creameries Products (Final Update). Accessed June 10, 2015.
69. Pushpangadan, P., and V. George. 2012. Basil*.* p. 55-72. *In* K.V. Peter (ed.), Handbook of Herbs and Spices, vol. 1. Woodhead Publishing Limited, Philadelphia, PA.
70. Quintavalla, S., and L. Vicini. 2002. Antimicrobial food packaging in meat industry*. Meat Science*. 62:373-380.
71. Rahman, M. S. 2007. Introduction to food preservation*. In* M.S. Rahman (ed.), Handbook of Food Preservation CRC Press, Boca Roca, Fl.
72. Rasato, A., C. Vitali, N. d. Laurentis, D. Amenise, and M. A. Nulillo. 2007. Antibacterial effect of some essential oils administered alone or in combination with norfloxacin*. Pytomedicine*. 104:727-732.
73. Rattanachaikunsopon, P., and P. Phumkhachorn. 2010. Potential of coriander (*Coriandrum sativum*) oil as a natural antimicrobial compound in controlling *Campylobacter jejuni* in raw meat*. Bioscience, Biotechnology, and Biochemistry*. 74:31-35.
74. Ravi, R., M. Prakash, and K. K. Bhat. 2007. Aroma characterization of coriander (*Coriandrum sativum L.*) oil samples*. European Food Research and Technology*. 225:367-374.

75. Reyes-Jurado, F., A. López-Malo, and E. Palou. 2016. Antimicrobial Activity of Individual and Combined Essential Oils against Foodborne Pathogenic Bacteria. *Journal of Food Protection*. 79:309-315.
76. Rico-Munoz, E., E. Bargiota, and P. Davidson. 1987. Effect of selected phenolic compounds on the membrane-bound adenosine triphosphatase of *Staphylococcus aureus. Food Microbiology*. 4:239-249.
77. Rocourt, J. 1996. Risk factors for listeriosis. *Food Control*. 7:195-202.
78. Scallan, E., R. M. Hoekstra, F. J. Angulo, R. V. Tauxe, M.-A. Widdowson, S. L. Roy, J. L. Jones, and P. M. Griffin. 2011. Foodborne illness acquired in the United States—major pathogens. *Emerg Infect Dis*. 17.
79. Shank, F. R., E. L. Elliot, I. K. Wachsmuth, and M. E. Losikoff. 1996. US position on *Listeria monocytogenes* in foods. *Food Control*. 7:229-234.
80. Sharma, M., and R. Sharma. 1999. Coriander. Handbook of herbs and spices. *Woodhead publishing ltd*. 246:1-6.
81. Shelef, L. A. 1984. Antimicrobial efffects of spices. *Journal of Food Safety*. 6:29-44.
82. Sikkema, J., J. De Bont, and B. Poolman. 1994. Interactions of cyclic hydrocarbons with biological membranes. *Journal of Biological Chemistry*. 269:8022-8028.
83. Silva, F., S. Ferreira, J. A. Queiroz, and F. C. Domingues. 2011. Coriander (*Coriandrum sativum L.*) essential oil: its antibacterial activity and mode of action evaluated by flow cytometry. *Journal of Medical Microbiology*. 60:1479-1486.
84. Simon, J., J. Quinn, and R. G. Murray. 1990. Basil: A source of essential oils. Timber Press, Portland, OR.
85. Skandamis, P. N., and G. J. E. Nychas. 2001. Effect of oregano essential oil on microbiological and physico-chemical attributes of minced meat stored in air and modified atmospheres. *Journal of Applied Microbiology*. 91.
86. Sokolovic, Z., A. Fuchs, and W. Goebel. 1990. Synthesis of species-specific stress proteins by virulent strains of *Listeria monocytogenes. Infection and Immunity*. 58:3582-3587.
87. Spencer, P. A., and G. Towers. 1988. Specificity of signal compounds detected by Agrobacterium tumefaciens. *Phytochemistry*. 27:2781-2785.
88. Stecchin, M. L., I. Sarais, and P. Giavedoni. 1993. Effect of essential oils on *Aeromonas hydrophila* in a culture medium and in cooked pork. *Journal of Food Protection*. 56:406-409.
89. Stopforth, J. D., P. N. Skandamis, P. M. Davidson, and J. N. Sofos. 2002. Naturally occuring compounds- Animal sources. *In* P.M. Davidson, J.N. Sofos, and A.L. Branen (ed.), Antimicrobial in Food. Taylor & Francis Group, Boca Raton, Fl.
90. Suppakul, P., J. Miltz, K. Sonneveld, and S. W. Bigger. 2003. Antimicrobial Properties of Basil and Its Possible Application in Food Packaging. *Journal of Agricultural and Food Chemistry*. 51:3197-3207.

91. Takahashi, H., M. Kashimura, H. Koiso, T. Kuda, and B. Kimura. 2013. Use of ferulic acid as a novel candidate of growth inhibiting agent against *Listeria monocytogenes* in ready-to-eat food*. Food Control*. 33:244-248.
92. Tarwadi, K., and V. Agte. 2003. Potential of commonly consumed green leafy vegetables for their antioxidant capacity and its linkage with the micronutrient profile*. International Journal of Food Sciences and Nutrition*. 54:417-425.
93. Techathuvanan, C., F. Reyes, J. R. D. David, and P. M. Davidson. 2014. Efficacy of Commercial Natural Antimicrobials Alone and in Combination against Pathogenic and Spoilage Microorganisms*. Journal of Food Protection*. 77:269-275.
94. Tsigarida, E., P. Skandamis, and G. J. E. Nychas. 2000. Behaviour of *Listeria monocytogenes* and autocthonous flora on meat stored under aerobic, vacuum and modified atmosphere packaging condition with or without the presence of oregano essential oil at 5C*. Journal of Applied Microbiology*. 89:901-909.
95. Ultee, A., M. H. J. Bennik, and R. Moezelaar. 2002. The phenolic hydroxyl group of carvacrol is essential against the food-borne pathogen *Bacillus cereus. Applied and Environmental Microbiology*. 68:1561-1568.
96. Ultee, A., E. Kets, and E. Smid. 1999. Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus. Applied and environmental microbiology*. 65:4606-4610.
97. Vigil, A. L., E. Palou, and S. M. Alzamora. 2005. Naturally occuring compounds- plant sources*.* p. 429-451. *In* P.M. Davidson, J.N. Sofos, and A.L. Branen (ed.), Antimicrobials in food Marcel Dekker.
98. Wen, A., P. Delaquis, K. Stanich, and P. Toivonen. 2003. Antilisterial activity of selected phenolic acids*. Food Microbiology*. 20:305-311.
99. Wendakoon, C. N., and M. Sakaguchi. 1993. Combined effect of sodium chloride and clove on growth and biogenic amine formation of *Enterobacter aerogenes* in mackerel muscle extract*. Journal of Food Protection*. 56:410-413.
100. Wilkins, K. M., and R. G. Board. 1989. Natural antimicrobial systems*. In* G.W. Gould (ed.), Mechanisms of action of food preservation procedures Elsevier Science Publishers New York.
101. Wilson, P., T. Brocklehurst, S. Arino, D. Thuault, M. Jakobsen, M. Lange, J. Farkas, J. Wimpenny, and J. Van Impe. 2002. Modelling microbial growth in structured foods: towards a unified approach*. International journal of food microbiology*. 73:275-289.
102. Zhou, F., B. Ji, H. Zhang, H. Jiang, Z. Yang, J. Li, J. Li, Y. Ren, and W. Yan. 2007. Synergistic effect of thymol and carvacrol combined with chelators and organic acids against *Salmonella Typhimurium. Journal of Food Protection*. 70:1704-1709.
103. Zink, D. L. 1997. The impact of consumer demands and trends on processing*. Emerging infectious diseases*. 3:467-469.

APPENDIX

Table 1. Design of Antimicrobial Combination Tests with Fractional Inhibitory Concentration Index and Interpretation of Effect

Antimicrobial MIC Proportion				FICI	Bacterial Growth Result	Effect
A	B	C	D			
Binary antimicrobial combination						
0	0	NA	NA	1	Growth	Control
1	0	NA	NA	1	One log reduction	MIC
0	1	NA	NA	1	One log reduction	MIC
3/4	1/4	NA	NA	1	Growth	Additive or antagonistic
					No log growth	Additive
1/2	1/2	NA	NA	1	Growth	Additive or antagonistic
					No log growth	Additive
1/4	3/4	NA	NA	1	Growth	Additive or antagonistic
					No log growth	Additive
1/4	1/4	NA	NA	0.5	Growth	Additive or antagonistic
					No log growth	Synergistic
3/4	3/4	NA	NA	1.5	Growth	Antagonistic
					No log growth	Additive or synergistic
Tertiary antimicrobial combinations						
1/3	1/3	1/3	NA	1	Growth	Additive or antagonistic
					No growth	Additive
1/2	1/4	1/4	NA	1	Growth	Additive or antagonistic
					No growth	Additive
1/4	1/2	1/4	NA	1	Growth	Additive or antagonistic
					No growth	Additive
1/4	1/4	1/2	NA	1	Growth	Additive or antagonistic
					No growth	Additive
1/6	1/6	1/6	NA	0.50	Growth	Additive or antagonistic
					No growth	Additive
1/2	1/2	1/2	NA	1.50	Growth	Antagonistic
					No growth	Additive or synergistic
Quaternary antimicrobial combinations						
1/4	1/4	1/4	1/4	1	Growth	Additive or Antagonistic
					No growth	Additive
1/2	1/6	1/6	1/6	1	Growth	Additive or Antagonistic
					No growth	Additive
1/6	1/2	1/6	1/6	1	Growth	Additive or Antagonistic
					No growth	Additive

Table 1 Continued. Design of Antimicrobial Combination Tests with Fractional Inhibitory Concentration Index and Interpretation of Effect

Antimicrobial MIC Proportion				FICI	Bacterial Growth Result	Effect
A	B	C	D			
Quaternary antimicrobial combination						
1/6	1/6	1/2	1/6	1	Growth	Additive or Antagonistic
					No growth	Additive
1/6	1/6	1/6	1/2	1	Growth	Additive or Antagonistic
					No growth	Additive
1/3	1/3	1/6	1/6	1	Growth	Additive or Antagonistic
					No growth	Additive
1/3	1/6	1/3	1/6	1	Growth	Additive or Antagonistic
					No growth	Additive
1/3	1/6	1/6	1/3	1	Growth	Additive or Antagonistic
					No growth	Additive
1/6	1/3	1/3	1/6	1	Growth	Additive or Antagonistic
					No growth	Additive
1/6	1/3	1/6	1/3	1	Growth	Additive or Antagonistic
					No growth	Additive
1/6	1/6	1/3	1/3	1	Growth	Additive or Antagonistic
					No growth	Additive
1/7	2/7	2/7	2/7	1	Growth	Additive or Antagonistic
					No growth	Additive
2/7	1/7	2/7	2/7	1	Growth	Additive or Antagonistic
					No growth	Additive
2/7	2/7	1/7	2/7	1	Growth	Additive or Antagonistic
					No growth	Additive
2/7	2/7	2/7	1/7	1	Growth	Additive or antagonistic
					No growth	Additive
1/8	1/8	1/8	1/8	0.50	Growth	Additive or antagonistic
					No Growth	Synergistic
3/8	3/8	3/8	3/8	1.50	Growth	Antagonistic
					No growth	Additive or synergistic

[a]*Synergistic, additive, and antagonistic effects of combined antimicrobials are defined by FICs* of ≤0.5, 1.0, and ≥1.5

[B]NA, not applicable

[C]No growth defined as <1 log cfu/ml

[D]Modified from Techathuvanan et al. *(93)*

Table 2. MICs[a] of Antimicrobials against *Listeria monocytogenes* Scott A at pH 6.0 and 25°C

	ΔLog at 48 h			
Control[b]	0 ppm			
	+2.70			
OEO[c]	200 ppm	250 ppm	312.5 ppm	624 ppm
	+1.80 log	-1.1 log	-2.43 log	-4.67 log
CEO	1666 ppm	2500 ppm	3333 ppm	5000 ppm
	+1.35 log	-1.27 log	-3.45 log	-4.83 log
BEO	2500 ppm	5000 ppm	7500 ppm	10000 ppm
	+0.66	+0.55	-1.12 log	-1.41 log
FA	1250 ppm	2500 ppm	5000 ppm	7500 ppm
	+1.71 log	+0.13 log	-1.18 log	-1.32 log

[a]MIC defined as a 1 log reduction

[b]Control – contained 1% DMSO; time 0, 4.75±0.29 CFU/ml, time 48 h, 7.45±0.03 CFU/ml

[b]OEO, Oregano Essential Oil; CEO, Coriander Essential Oil; BEO, Basil Essential Oil; FA, Ferulic Acid

Table 3. Effect of Binary Combinations on *Listeria monocytogenes* Scott A at pH 6.0 and 25°C

Antimicrobial Combination														
Antimicrobial MIC proportion			OEO+BEO		OEO+CEO		OEO+FA		CEO+BEO		CEO+FA		BEO+FA	
FIC	A	B	Avg log 0 h	Avg log 48 h	Avg log 0 h	Avg log 48 h	Avg log 0 h	Avg log 48 h	Avg log 0 h	Avg log 48 h	Avg log 0 h	Avg log 48 h	Avg log 0 h	Avg log 48 h
1	3/4	1/4	4.69±0.18	<1	3.91±0.05	<1	3.63±0.07	3.25±0.02	3.53±0.19	<1	4.27±0.09	2.44±0.35	4.36±0.22	<1
1	1/2	1/2	4.71±0.12	<1	3.28±0.01	<1	4.31±0.04	3.63±0.07	3.72±0.20	<1	4.70±0.00	2.01±0.12	4.29±0.08	<1
1	1/4	3/4	4.84±0.09	<1	3.35±0.05	<1	4.60±0.04	3.28±0.02	3.90±0.21	<1	3.51±0.06	1.72±0.08	4.13±0.23	<1
0.5	1/4	1/4	4.94±0.06	<1	3.96±0.10	<1	4.58±0.02	5.03±0.10	3.82±0.19	<1	4.30±0.05	3.08±0.08	3.95±0.23	<1
1.5	3/4	3/4	4.49±0.14	<1	2.82±0.01	<1	2.72±0.03	<1	3.46±0.22	<1	4.76±0.03	1.92±0.86	4.34±0.27	<1

[a]MIC starting concentration 250 ppm OEO, 2500 ppm CEO, 7500 ppm BEO, and 5000 ppm FA

[b]OEO, Oregano Essential Oil; CEO, Coriander Essential Oil; BEO, Basil Essential Oil; FA, Ferulic Acid

Table 4. Effect of Tertiary Combinations on *Listeria monocytogenes* Scott A at pH 6.0 and 25°C

Antimicrobial Combinations											
Antimicrobial MIC Proportion				OEO+CEO+BEO		OEO+CEO+FA		OEO+BEO+FA		CEO+BEO+FA	
FIC	A	B	C	Avg log 0 h	Avg log 48 h	Avg log 0 h	Avg log 48 h	Avg log 0 h	Avg log 48 h	Avg log 0 h	Avg log 48 h
1	1/3	1/3	1/3	4.53±0.04	<1	3.98±0.06	<1	5.06±0.35	1.50±1.48	4.77±0.02	<1
1	1/2	1/4	1/4	4.49±0.00	<1	4.11±0.31	0.55±0.95	4.95±0.07	1.07±1.02	4.33±0.47	<1
1	1/4	1/2	1/4	4.24±0.14	<1	3.86±0.25	<1	4.86±0.17	1.36±1.57	4.49±0.08	<1
1	1/4	1/4	1/2	4.62±0.00	<1	4.15±0.40	1.71±1.88	5.07±0.20	1.56±1.47	4.70±0.03	<1
0.5	1/6	1/6	1/6	4.73±0.01	<1	4.71±0.29	4.62±0.00	5.02±0.10	1.22±1.89	4.73±0.01	<1
1.5	1/2	1/2	1/2	4.45±0.04	<1	3.47±0.03	<1	4.91±0.11	<1	4.47±0.16	<1

[a]MIC starting concentration 250 ppm OEO, 2500 ppm CEO, 7500 ppm BEO, and 5000 ppm FA

[b]OEO, Oregano Essential Oil; CEO, Coriander Essential Oil; BEO, Basil Essential Oil; FA, Ferulic Acid

Table 5. Effect of Quaternary Combinations on *Listeria monocytogenes* Scott A at pH 6.0 and 25°C

Antimicrobial MIC Combinations						
FIC	OEO+CEO+Basil+FA				Avg log 0 h	Avg log 48 h
1	1/4	1/4	1/4	1/4	4.98±0.04	<1
1	1/2	1/6	1/6	1/6	4.88±0.10	<1
1	1/6	1/2	1/6	1/6	4.53±0.04	<1
1	1/6	1/6	1/2	1/6	4.58±0.17	<1
1	1/6	1/6	1/6	1/2	4.72±0.03	<1
1	1/3	1/3	1/6	1/6	4.58±0.15	<1
1	1/3	1/6	1/3	1/6	4.64±0.22	<1
1	1/3	1/6	1/6	1/3	4.8±0.16	<1
1	1/6	1/3	1/3	1/6	4.68±0.03	<1
1	1/6	1/3	1/6	1/3	4.53±0.04	<1
1	1/6	1/6	1/3	1/3	4.82±0.13	<1
1	1/7	2/7	2/7	2/7	4.84±0.15	<1
1	2/7	1/7	2/7	2/7	4.88±0.15	2.13±0.62
1	2/7	2/7	1/7	2/7	4.42±0.01	<1
1	2/7	2/7	2/7	1/7	4.75±0.11	<1
0.5	1/8	1/8	1/8	1/8	4.78±0.11	<1
0.25	1/16	1/16	1/16	1/16	5.06±0.05	2.60±0.01
0.125	1/32	1/32	1/32	1/32	5.08±0.03	4.20±0.02
1.5	3/8	3/8	3/8	3/8	4.62±0.09	<1

[a]MIC starting concentration 250 ppm OEO, 2500 ppm CEO, 7500 ppm BEO, and 5000 ppm FA

[b]OEO, Oregano Essential Oil; CEO, Coriander Essential Oil; BEO, Basil Essential Oil; FA, Ferulic Acid

Table 6. Interpretation of Effects of Combined Antimicrobials against Listeria monocytogenes Scott A at pH 6.0 and 25°C

Antimicrobial Combination	Effect of Combined Antimicrobial
OEO+BEO	Synergistic
OEO+CEO	Additive
OEO+FA	Additive
CEO+BEO	Synergistic
CEO+FA	Synergistic
BEO+FA	Synergistic
OEO+CEO+BA	Synergistic
OEO+CEO+FA	Additive
OEO+BEO+FA	Synergistic
CEO+BEO+FA	Synergistic
OEO+CEO+BEO+FA	Synergistic

[a] Synergistic, additive, and antagonistic effects of combined antimicrobials defined by FICs of ≤0.5, 1.0, and >1.5, respectively.

[b] OEO, Oregano Essential Oil; CEO, Coriander Essential Oil; BEO, Basil Essential Oil; FA, Ferulic Acid

VITA

Savannah Grace Hawkins, the daughter of Bill and Pam Hawkins, was born on November 27, 1991 in Chattanooga, Tennessee. Savannah grew up in Murfreesboro, Tennessee and graduate from Siegel High School in 2010. She continued her education at the University of Tennessee where she graduated with a B.S in Food Science and Technology in 2014. After an internship at McKee foods, Savannah returned to the University of Tennessee and completed her Master's degree in Food Science and Technology with a focus in Food Microbiology.