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Antilisterial Characteristics of Volatile Essential Oils

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ANTILISTERIAL CHARACTERISTICS OF VOLATILE ESSENTIAL OILS

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THESIS

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A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
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at the University of Kentucky

By

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ABSTRACT OF THESIS

ANTILISTERIAL CHARACTERISTICS OF VOLATILE ESSENTIAL OILS

This study explored the *in vitro* and *in situ* antilisterial inhibitory activity of 16 essential oils during indirect exposure: Spanish Basil oil (*Ocimum basilicum*), Bay oil (*Pimenta racemosa*), Italian Bergamot oil (*Citrus bergamia*), Roman Chamomile oil (*Anthemis nobilis*), Sri Lanka Cinnamon oil (*Cinnamomum zeylanicum*), Citral, Clove Bud oil (*Syzygium aromaticum*), Cumin Seed oil (*cuminum cyminum*), Eucalyptus oil (*Eucalyptus globulus*), Eugenol, Geranium extract (*Pelargonium graveolens*), Marjoram oil (*Origanum majorana*), Neroli extract (*Citrus aurantium*), Peppermint oil (*Mentha piperita* L.), Rosemary oil (*Rosmarinus officinalis* L.),and Spanish Sage oil (*Salvia officinalis* L.).   All essential oils were tested against *Listeria monocytogenes* (ATCC 4644). *In vitro* inhibitory activity was determined using the microatmosphere method at three temperatures (37°C, 24°C, 4°C) and six possible volumes (0, 10µl, 25µl, 50µl, 100µl, 150µl, or 200µl). *In situ* inhibitory activity was determined using inoculated bologna slices packaged in Modified Atmosphere Packaging (80% O_2, 20% CO_2). Essential oils (0, 0.13ml, 1.35ml, or 2.70ml) were injected into the sample packages adjacent, but not touching, the bologna slices and stored at 24°C for 24h. Basil oil displayed the least antilisterial activity across the three temperature applications *in vitro*. Peppermint, Cumin Seed, and Citral consistently exhibited the greatest antilisterial activity among the temperature applications *in vitro*. However, only Eugenol applied at 1.35ml achieved a mean one $\log_{10}$ CFU/ml reduction in *LM in situ*, which could not be replicated. Peppermint (P < 0.048) displayed significant differences between application volumes (0.13ml, 1.35ml) but did not attain a mean one $\log_{10}$ CFU/ml reduction in *LM*. This study suggests that while various essential oils can display antilisterial activity *in vitro*, transitioning into a MAP food system warrants further research in mode of actions and application volumes.

KEYWORDS: Essential Oil, Microatmosphere, Modified Atmosphere Packaging, Bologna, *Listeria monocytogenes*

____Leeann Slaughter____

_____April 25th, 2013_____

ANTILISTERIAL CHARACTERISTICS OF VOLATILE ESSENTIAL OILS

By

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_____April 25th, 2013_____
(Date)

To Lindsey and Jennifer,

Thank you for your encouragement, understanding, and guidance throughout my life.

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CHAPTER 1

INTRODUCTION

Listeria monocytogenes is a Gram-positive bacterium known for causing foodborne Listeriosis in humans. Traditionally, ready-to-eat foods such as deli meats, salads, and dairy products have been identified as contamination sources due to the lack of heating before consumption (Silk, Date et al. 2012). Since 1996, the Center for Disease Control Foodborne Diseases Active Surveillance Network, known as Foodnet, has compiled information on clinical foodborne outbreaks in roughly 15% of the United States population in over ten states (CDC 2011a). Reports show that while the clinical incidence of *Listeria* has decreased by 38% since 1996, serious Listerial illness is highest among the six common pathogens (*Campylobacter, Salmonella*, STEC 0157, *Yersinia, Listeria, Vibrio*), reporting 90% hospitalization in confirmed cases, as well as displaying a high case-fatality ratio, 13% (CDC 2011b). Only nontyphoidal *Salmonella* and *Toxoplasma gondii* as reported by the CDC are responsible for more foodborne illness deaths than *Listeria* (Scallan, Hoekstra et al. 2011). Listeriosis manifests as febrile gastroenteritis, meningitis, fetal abortion, septicemia, or encephalitis. Immunocompromised, elderly, or pregnant individuals represent the highest risk groups for Listeriosis, which can progress from a intestinal infection to lethal neural or blood infections based on the strength of human immune responses (Hamon, Bierne et al. 2006). Due to the high fatality of Listeriosis, the Food and Drug Administration (FDA) and United States Department of Agriculture (USDA) have improved policies and

guidance of preventing *L. monocytogenes* contamination in food products, namely ready-to-eat food (USDA-FSIS 2001).

Ready-to-eat (RTE) foods are products which do not require a heating step before consumption, e.g. cheese, delicatessen meats, milk, as well as various fruits and vegetables (NACMCF 2005). RTE products are susceptible to Listeria contamination due to its ability to survive and grow at refrigeration temperatures and within vacuum packaging. *L. monocytogenes* can be eliminated by post-packaging pasteurization, antimicrobial compounds such as sodium diacetate and lactate, and general sanitation procedures. However, RTE meat quality (color, texture, taste) is affected by additional cooking or ingredients and can affect the likelihood of a product being purchased by consumers (USDA-FSIS 2003). In addition, RTE meats can become contaminated after cooking and before packaging, despite these steps typically functioning as barriers to bacterial growth (Ingham, Buege et al. 2004).

Promising research to inhibit or eliminate *L. monocytogenes* in packaged RTE meats is the incorporation of essential oils in processing. Essential oils are the volatile and non-volatile chemical constituents obtained from whole plants and plant parts, such as stems, leaves, or roots, by steam distillation. Minor constituent ratios vary among the same essential oil due to species, geographical location, harvest season, and other environmental and genetic differences (Smith, Cohen et al. 2005, Hussain, Anwar et al. 2008). The oil constituents are largely responsible for the aroma and taste associated with specific plants, and some constituents are shown to display antioxidant and antimicrobial functionality (Bozin, Mimica-Dukic et al. 2006, Celiktas, Kocabas et al. 2007). As such, a wide range of plant essential oils have been applied to *L. monocytogenes in vitro* as

well as in packaged products to study the antimicrobial relevance of essential oils in the prevention of Listeriosis (Lis-Balchin and Deans 2003, Oussalah, Caillet et al. 2007, Dorman and Deans 2008). Increased research efforts have studied the direct application of essential oils by dipping, mixing, or spraying the oil directly on a food product or lab culture (Lis-Balchin and Deans 2003, Busatta, Vidal et al. 2008, De Azeredo, Stamford et al. 2011). A smaller area of research has explored using the application of vapor phases, or the volatility of an essential oil, in indirect antimicrobial effects (Goñi, López et al. 2009, Nedorostova, Kloucek et al. 2009).

The purpose of this research was to explore the *in vitro* and *in situ* use of 16 essential oils for the indirect volatile inhibition of *L. monocytogenes*. In the first experiment, the inhibition activity of the selected essential oils against *Listeria monocytogenes* (LM) was assessed *in vitro* using agar Petri dish cultures. Agar dishes were inoculated with LM and a filter paper was attached to the inside lid of the dish, on which an essential oil was applied. Qualitative inhibition was determined visually. Three temperatures (37°C, 24°C, 4°C) were used to view how essential oil inhibition activity differed in three separate storage situations. The second experiment was *in situ*: a large package was used to study the effect of increased space on the bacteriostatic ability of the essential oils in a RTE meat system. In this experiment, oil was applied as a liquid adjacent to the inoculated agar plate or food product, taking care that the oil was not touching the inoculated food sample. After determining the smallest practical volume of each essential oil needed for inhibition *in vitro*, a range of volumes were exposed to sliced bologna in a high oxygen (80%) Modified Atmosphere Packaging system. Because commercial bologna contains an anti-listerial component required by the USDA, e.g.

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sodium diacetate or lactate, the bologna used in this experiment was formulated in the University of Kentucky Meats Laboratory (Lexington, KY) without anti-listerial ingredients to more closely determine the effects of our essential oils. The objectives of this thesis research were:

1. To quantify the *in vitro* bacteriostatic ability of essential oils on *L. monocytogenes* using an agar plate at different volumes and storage temperatures to determine if essential oil potency varies among food storage temperatures, as well as define the minimum volume needed for listerial inhibition.

2. To quantify the *in situ* bacteriostatic ability of essential oils on *L. monocytogenes* in a packaged RTE meat, using sliced bologna held at room temperature for 24h in high oxygen (80% O_2, 20% CO_2) Modified Atmospheric packaging to determine changes in the antilisterial activity of essential oils when scaled for larger application in food.

CHAPTER 2

LITERATURE REVIEW

2.1. Ready-to-Eat Meats

A Ready-to-Eat (RTE) meat is defined by 9 CFR (Code of Federal Regulations) Part 430 as, "A meat or poultry product that is in a form that is edible without additional preparation to achieve food safety and may receive additional preparation for palatability or aesthetic, epicurean, gastronomic, or culinary purposes. RTE product is not required to bear a safe-handling instruction (as required for non-RTE products by 9 CFR 317.2(l) and 381.125(b)) or other labeling that directs that the product must be cooked or otherwise treated for safety, and can include frozen meat and poultry products. (USDA-FSIS 2001)"

RTE meats can be either non-shelf-stable, requiring refrigeration during storage to inhibit the growth of spoilage organisms and pathogens, or shelf-stable, as they can be stored at room temperature (USDA-FSIS 2001). RTE meats are categorized by specific processing into five types after separation by shelf-stability: dried products; salt-cured products; fermented products; cooked or otherwise processed whole and comminuted products; and thermally-processed, commercially sterile products (USDA-FSIS 2001). Dried products are shelf-stable meats and poultry that have undergone dehydration processing to reduce water activity until foodborne pathogens cannot survive, but do not require fermentation or lethality cooking. Water activity (a_w) is a measurement of unbound or free water in a food that can be utilized by bacteria for growth, as opposed to moisture content, which is the total moisture percentage contained in a food product.

Pure water has a a_w of 1.00, while fresh meat generally contains a a_w of 0.98 (USDA-FSIS 2001). The Centers for Disease Control and Prevention (CDC) recommendation for beef jerky processing, for example, is to dry the product at a high temperature, above 155°F, for four hours before dropping the temperature range to above 140°F for an additional four hours, which will yield an approximate a_w of 0.86 (USDA-FSIS 2001). Salt-cured products are shelf-stable meats and poultry that rely on the application of salt to reduce a_w and inhibit microbial growth without intensive heat treatments, but can also contain nitrites and smoke processing to inhibit pathogens, e.g. country hams, dry-cured duck, and prosciutto (USDA-FSIS 2001). Fermented products are shelf-stable meats and poultry which rely on some type of acid formation to inhibit pathogens through lowered pH, either through lactic acid-forming homofermentative starter cultures, such as *Lactobacillus plantarum*, or direct application of citric acid (Acton 1977). Semi-dry fermented meat, such as Lebanon bologna, retains between 40%-50% moisture (a_w 0.93) while achieving a pH range of 4.7-4.9, and though it can be fully cooked during processing, it is not shelf-stable and requires refrigeration due to the high moisture content (Acton 1977). On the other hand, dry fermented meats, such as pepperoni, retain an average of 35% moisture (a_w 0.89) and a final pH of 4.9-5.1, do not require cooking, typically require a longer processing time, and are shelf-stable (Acton 1977). Thermally-processed, commercially sterile meats such as canned spaghetti and meatballs have been subjected to pathogen lethality cooking after packaging in hermetically sealed vessels, are shelf-stable despite having an a_w above 0.85, and are considered to be at low risk for bacterial contamination (USDA-FSIS 2001). In contrast, cooked or otherwise processed whole and comminuted products, such as bologna and roast beef, are products that are

processed as whole muscles, chopped and ground, or restructured and shaped before a pathogen lethality cooking step occurs; the product is usually packaged after the cooking step, with no bactericidal step after packaging in the event of bacterial contamination after cooking. These products are non-shelf-stable, and are the primary target for *Listeria monocytogenes* contamination in RTE meats (USDA-FSIS 2003, Ingham, Buege et al. 2004).

RTE foods were first reliably identified as a source of *L. monocytogenes* contamination for humans in 1981. A large outbreak of Listeriosis occurred in Canada due to consumption of refrigerated coleslaw, which had been prepared with cabbage from a regional producer known to have reported cases of Listeriosis in sheep (Schlech, Lavigne et al. 1983). The serotype of *L. monocytogenes* grown from the suspicious coleslaw matched the serotype isolated from a patient's blood, and epidemiological dots were connected (Schlech, Lavigne et al. 1983). Epidemiological tracing of foodborne illness resulting from *L. monocytogenes* consumption went on to suggest outbreak sources from undercooked hot dogs and chicken in both the U.S. and Canada (Schwartz, Ciesielski et al. 1988). The serious fatalistic nature of this foodborne illness, discussed in proceeding sections, drew attention from the United States Department of Agriculture (USDA) which resulted in increased processing regulations for RTE foods. However, despite increased processing regulation periodic outbreaks from delicatessen meat, cheeses, vegetables and fruits continue to occur (MacDonald, Whitwam et al. 2005, Mead, Dunne et al. 2006, CDC 2012). The ubiquitous nature of *Listeria monocytogenes* in food systems has prompted countless research avenues toward understanding the

genetic and physiological history, survival, pathogenicity, inhibition and elimination of the organism.

2.2. *Listeria monocytogenes*

L. monocytogenes (*LM*) is a non-spore forming, facultatively anaerobic, Gram-positive saprophyte rod bacterium occurring in soil, surface water, silage, sewage and slaughterhouse waste, bovine milk, human and animal hosts, and food processing facilities (Weis and Seeliger 1975, McCarthy 1990, Farber and Peterkin 1991, Thevenot, Dernburg et al. 2006, Lecuit 2007). *LM* has been long known to possess a wide growth range and thermal tolerance of -0.4°C to 50°C (Walker and Stringer 1987, Junttila, Niemela et al. 1988). However, the peritrichous flagella produced on the cell surface of *L. monocytogenes* that provide tumbling motility thrive in a narrow growth range between 20°C and 25°C, dropping off in production at incubation temperatures (37°C) (Peel, Donachie et al. 1988). *LM* has been able to persist in a variety of environments and temperatures, and some strains have exhibited a tendency to establish and persist for months or years in environmental stresses, such as fluctuating pH and the presence of metal ions, due to flexible gene expressions and attaining replicons from hardy staphylococci strains (Lemaitre, Echchannaoui et al. 1998, Lunden, Autio et al. 2002, Chaturongakul, Raengpradub et al. 2008). Tolerance of transitioning between oxygen-rich and low-oxygen environments, such as those present in RTE meat processing and packaging, also enable *L. monocytogenes* to survive in food systems (Lungu, Ricke et al. 2009). The broad growth and survival conditions of *L. monocytogenes*, with the exception of pasteurization heat, make the bacteria a continuous risk for foodborne illness.

2.2.1. Listeriosis. The disease Listeriosis is a direct result of consuming foods contaminated with *Listeria monocytogenes*, a pathogen which transitions from a saprophyte to a cytosolic bacteria upon consumption due to nutrient availability cues (Freitag, Port et al. 2009). Listeriosis is known for causing serious illness primarily in the immunocompromised, the elderly, newborns, and pregnant women. Clinical symptoms in these high-risk groups of individuals include meningitis, encephalitis, maternal-fetal infection and septicemia, though healthy individuals may express febrile gastroenteritis due to mucosal inflammation possibly caused by pathogen entry into the host intestinal mucosa (Ooi and Lorber 2005, Hamon, Bierne et al. 2006). Hospitalization rates in the U.S. due to Listeriosis infection is approximately 90% of confirmed cases, and the 13 % case-fatality ratio is surpassed by only *Salmonella* and *Toxoplasma gondii* among 31 major foodborne pathogens (Scallan, Hoekstra et al. 2011, CDC 2011b).

The high virulence capacity of *L. monocytogenes* to manifest various modes of disease lies in its ability to cross three human host barriers after ingestion: the intestinal barrier, the blood-brain barrier, or the fetoplacental barrier (Khelef, Lecuit et al. 2005). First, the intestinal epithelium barrier is breached by one of two mechanisms. The primary mechanism involves specific internalins, an isoform of receptor molecules recognized by *L. monocytogenes* molecules, which are located on the enterocytes occurring in the epithelium lining of intestinal microvilli of humans (Vázquez-Boland, Kuhn et al. 2001). *L. monocytogenes* accesses the epithelium using internalin surface proteins InlB, InlC, and InlJ to bind to a human mucin protein isoform, human Muc2, unlike most pathogens which produce general mucinases to penetrate the mucosal lining (Linden, Bierne et al. 2008, Sleator, Watson et al. 2009). InlA then binds E-cadherin, an

intestinal adhesion cell, while InlB binds a hepatocyte growth factor receptor, HGF, allowing *L. monocytogenes* to establish in intestinal host cells (Pizarro-Cerda and Cossart 2006). In additional contrast to other gastrointestinal pathogens, *L. monocytogenes* only uses flagella for motility, not as an adhesion or secretion apparatus (O'Neil and Marquis 2006). The second mechanism incorporates nonspecific translocation of pathogens across the M-cell epithelium overlying Peyer's patches, which occurs in hosts not possessing the specific isoforms described in the former mechanism (Vázquez-Boland, Kuhn et al. 2001, Corr, Hill et al. 2006). Of the aforementioned mechanisms, the specific binding of internalins appears to be both more efficient and more common in *L. monocytogenes* human host pathogenesis, and both mechanisms require the pathogen to be encased in a phagocyte vacuole to penetrate the intestinal barrier (Schnupf and Portnoy 2007, Schuppler and Loessner 2010). To escape the phagocyte vacuole after entering host cells, *L. monocytogenes* produces a pore-forming hemolysin, listeriolysin O (LLO), as well as two phospholipases, PI-PlcA and PI-PlcB, which are activated by a metalloprotease (Schnupf and Portnoy 2007, Scortti, Monzo et al. 2007, Kuhn, Scortti et al. 2008). Upon release, pathogen replication takes place through acquisition of the host's nutrients, namely hexose phosphate sugars, lipoic acid and peptides (O'Riordan, Moors et al. 2003, Joseph and Goebel 2007, Kuhn, Scortti et al. 2008). At that time, the pathogens move through the host cytoplasm by the expression of ActA, a surface protein which induces the polymerization of small g-actin globules into longer f-actin filaments, effectively propelling *L. monocytogenes* toward the cytoplasmic membrane of adjacent cells (Pizarro-Cerda and Cossart 2006). Once the replicated pathogens, dubbed listeriapods, reach the neighboring cells and are endocytosed through the cell membrane they repeat

the secretion of LLO and phospholipase, in this case phosphatidylcholine phospholipase (PC-PLC), to liberate themselves from secondary vacuoles and continue to spread cell-to-cell, eluding adaptive host immune system responses and potentially causing febrile gastroenteritis in otherwise healthy adults (Vazquez-Boland, Kocks et al. 1992, Yeung, Na et al. 2007, Schuppler and Loessner 2010). Once translocated across the human intestinal barrier *L. monocytogenes* moves to the liver, spleen, and mesenteric lymph nodes (Lecuit 2005). However, the exact mechanisms responsible for *L. monocytogenes* translocation from the human intestine through blood, brain and placental barriers are less concrete, due to physiological and biochemical variation among species of animal models (Vázquez-Boland, Kuhn et al. 2001, Hoelzer, Pouillot et al. 2012). Based on current animal models, *L. monocytogenes* collects in the aforementioned organs through infected dendrites and macrophages and elicits an adaptive immune response from the host. If the immune response is unsuccessful, *L. monocytogenes* is then capable of directly invading neurons and endothelium cells of blood vessels and will continue to travel through the blood-brain barrier and placental barrier through infected macrophages (Wilson and Drevets 1998, Vázquez-Boland, Kuhn et al. 2001, Hoelzer, Pouillot et al. 2012). After *L. monocytogenes* crosses the blood-brain and placental barriers, immune-compromised or pregnant individuals and fetuses manifest high-mortality invasive listeriosis through meningitis, encephalitis, septicemia, abortions, and stillbirths (Hamon, Bierne et al. 2006). Though rates of Listeriosis have declined in the U.S., the high rate of fatalities in immune-compromised individuals has led to increased research areas and regulation of possible consumer exposure sources of *L. monocytogenes*. The USDA has adopted a "zero tolerance" policy for the pathogen in RTE meats (Ooi and Lorber 2005, Liu 2008).

2.2.2. Listeriosis prevention. The prevention of L. monocytogenes contamination in a food processing plant varies by the quality potential (color, texture, taste, storage type) of the food in question. Many protocols are emerging to enhance product safety, quality, and consumer appeal while maintaining various levels of individuality between processing types (thermal parameters, time, physical manipulation, ingredient formulation). Currently, three points of control are recognized in contamination prevention: plant sanitation, antimicrobial ingredient formulation and food environment, and post-package decontamination (Zhu, Du et al. 2005). While plant sanitation procedure is the first and foremost control step to prevent microbial growth and contamination, additional steps in bacterial growth prevention are often needed to ensure a safe food.

Chemical anti-listerial additives such as sodium diacetate, sodium lactate, and trisodium phosphate have been largely incorporated in commercial RTE meat products due to positive validation studies with minimal quality faults (Mbandi and Shelef 2002, Stekelenburg 2003, Zhu, Du et al. 2005). Antimicrobial ingredients typically inhibit the growth of pathogens by either reducing the pH of the product or disrupting the cell membrane of the bacteria (Apostolidis, Kwon et al. 2008). Biopreservation, the use of beneficial or neutral bacteria to inhibit the growth of pathogenic bacteria, offers an alternative processing method to organic or inorganic salts like sodium diacetate. Commercial cultures used in the fermentation and curing of food can produce lactic acid, which is shown to inhibit the growth of L. monocytogenes (Zhu, Du et al. 2005). Microbe strains of *Lactobacillus, Pediococcus, Bifidobacteria* and *Enterococcus* can produce bacteriocins such as nisin, reuterin or enterocin, which are ribosomally

synthesized polypeptides able to inhibit or eliminate similar bacteria through membrane disruption (Cotter, Hill et al. 2005, Zhu, Du et al. 2005). The exact mode of action is theorized, but peptide interaction with the targeted bacterial cell receptors to result in cell death, binding with the outer membrane to enhance permeabilization, or ion-mediated translocation of the peptide into the bacterial cell to disrupt functional processes are all viable explanations (Thennarasu, Lee et al. 2005). In both cases, bacteria can be used directly in the food product or separately grown for their bacteriocins, which are then added to the product. While most food additives are focused on the inhibition of *L. monocytogenes*, with the exception of some bactericidal bacteriocins, post-package decontamination procedures target the eradication of the bacteria. Post-package decontamination currently encompasses the use of in-package thermal pasteurization, irradiation, and high-pressure processing (Zhu, Du et al. 2005). In-package thermal pasteurization uses heat to distress and kill *L. monocytogenes*, which can interact with the texture, color, water-holding capacity, and other quality aspects of a product (Zhu, Du et al. 2005). Irradiation, however, utilizes ionizing radiation to degrade molecular bonds within the bacterial cell to kill the pathogen with minimal food quality changes (Lacroix and Ouattara 2000). Lastly, the most recent technological development in bactericidal procedure is high-pressure processing. This method uses immense non-thermal pressure to burst microbial cell walls with various changes in quality aspects of a food, such as protein denaturation creating soft or hard texture (Hugas, Garriga et al. 2002). At high pressures (150 MPa) meat color can undergo changes similar to cooked meat, while pressures below 200 MPa have shown to increase meat tenderness (Hugas, Garriga et al. 2002). The use of any one technique to inhibit or kill L. monocytogenes in a RTE food

ideally should harmonize with desired quality changes; ultimately, however, a method must be employed per USDA regulation.

The USDA mandates three broad legal options for use in a food processing plant to control and/or prevent *L. monocytogenes* in RTE products: Alternative One, Two, or Three. Alternative One involves the use of both a post-lethality treatment and a known growth inhibitor, such as in-package thermal pasteurization coupled with the use of sodium diacetate as a food ingredient (USDA-FSIS 2003). A product processed under Alternative One is considered the lowest risk for *L. monocytogenes* contamination, and requires the lowest level of microbial testing and regulation (USDA-FSIS 2003). Validation of the antilisterial effectiveness of the processes are recorded in the processors' Hazard Analysis and Critical Control Points (HACCP) document and microbial testing is not mandatory if the conditions for LM lethality are met (Firouzi, Shekarforoush et al. 2007). Alternative Two employs either the use of a post-lethality treatment or a known growth inhibitor, such as incorporating a mixture of sodium diacetate, lactate, and phosphate into an uncooked product that will not receive a post-package thermal treatment. This procedure is subject to more microbial testing and sanitation documentation due to a slight increased risk of *Listeria* contamination in comparison to Alternative One (USDA-FSIS 2003). The finished product must be tested for LM if using an antimicrobial agent, but the FSIS will likely not conduct validation tests if the establishment has records test results (Firouzi, Shekarforoush et al. 2007). Alternative Three relies on a sanitation protocol as the only control for *L. monocytogenes* contamination (USDA-FSIS 2003). Processing plants operating under Alternative Three are subject to more intensive scrutiny and USDA inspection activity due to a higher

perceived increase risk for *L. monocytogenes* contamination in comparison to Alternatives One and Two (USDA-FSIS 2003). Facilities must test food contact surfaces for LM as part of sanitation management and justify a testing frequency sufficient to prevent LM (Firouzi, Shekarforoush et al. 2007). As new packaging and manufacturing technologies emerge in RTE meats which must adhere to legal protocol, new anti-listerial compounds have become a research area, encompassing both synthetic and naturally collected chemicals to inhibit *L. monocytogenes in vitro* and *in situ*.

2.3. Essential Oils

In recent decades, essential oils extracted through steam distillation have been explored for anti-listerial properties in a variety of processing techniques. Almost all parts or organs of plants generate secondary metabolites for defensive, reproductive, or survival purposes called essential oils (Bakkali, Averbeck et al. 2008). The distinctive oily, volatile and fragrant metabolite characterization was first termed an essential oil by Paracelsus von Hohenheim in the 16th century as a "Quinta essential" component of a drug (Guenther 1950). It is widely believed the medicinal, aromatic, and food preservation application of essential oils have been used since ancient times; ancient Egyptians reportedly incorporated aromatic plants, primarily for the essential oils, in embalming practices to slow the growth of bacteria and rate of decay (Edris 2007). Though oils can be released through grinding, pressing and heating, the extraction of essential oils using steam, termed hydro-distillation, was first recorded by Arabic people in the Middle Ages (Bakkali, Averbeck et al. 2008). Other methods of extraction now include utilization of liquid carbon dioxide, supercritical carbon dioxide, lipophilic

solvents, microwaves and low or high pressure distillation (Bakkali, Averbeck et al. 2008).

The number of components in an essential oil can range from 20-60, though most constituents have representative major and minor concentration groups by which essential oils are categorized. For example, carvacrol and thymol are typically present in high concentrations of *Origanum compactum*, commonly known as oregano, while 1,8-cineole is the most prevalent component of *Cinnamomum camphora*, known as camphor oil (Bakkali, Averbeck et al. 2008). Exact chemical compositions of the same essential oil type can be dissimilar due to the environmental fluctuation, soil composition, and species of plant, as well as the chemical or physical nature of extraction (Masotti, Juteau et al. 2003, Angioni, Barra et al. 2006). Due to the slight variations in extracted essential oils, pharmaceutical and fragrance companies often assess chemical composition using gas chromatography, mass spectrometry, or similar validation testing. Essential oil components are categorized into one of two types of plant metabolites regardless of major or minute concentrations: terpenes and metabolites of terpenoid origin or aromatic and aliphatic compounds (Faleiro 2011).

Terpenes are differentiated by an array of structural types and compounds, monoterpenes (C10), sesquiterpenes (C15) and diterpenes (C20) being the most commonly metabolized plant products (Degenhardt, Köllner et al. 2009). The less commonly produced of the two types are aromatic metabolites generated from phenylpropanes and are sometimes simply referred to as the phenypropene group of essential oils; common phenylpropanoids identified in essential oils include cinnamaldehyde, eugenol, vanillin, and safrole (Miguel, Cruz et al. 2010, Hyldgaard,

Mygind et al. 2012). Though phenylpropanoids are less common in essential oils, they have been researched to show high antibacterial activity (Bakkali, Averbeck et al. 2008, Dorman and Deans 2008, Bassolé and Juliani 2012). Oxygenated terpenoids, alcohols and phenolic terpenes are often found to have antimicrobial properties, and the interaction of specific constituents such as carvacrol and thymol has been suggested to inhibit, enhance, or complement antimicrobial activity (Davidson and Parish 1989, Burt 2004, Bakkali, Averbeck et al. 2008). In addition, some whole essential oils have exhibited elevated antibacterial effects in comparison to their major constituents alone, suggesting a presence and interaction of minor constituents is sometimes needed to enhance the antimicrobial use of essential oils, called synergism (Bassolé and Juliani 2012, Hyldgaard, Mygind et al. 2012). It is important to note that some constituents and whole essential oils can be used separately to prevent deterioration and extend the edible time-table of foods by slowing the growth of decomposition bacteria, while not being particularly useful in the inhibition or elimination of pathogenic bacteria alone (Lanciotti, Corbo et al. 1999, Guillén, Zapata et al. 2007). This distinction is due to a number of theorized and confirmed mechanisms by which essential oils and individual constituents interact with, inhibit, or eliminate microorganisms.

  2.3.1. Modes of Action. Essential oils have been researched to induce either bactericidal or bacteriostatic activity on a range of microorganisms depending on specific mechanistic interactions (Dorman and Deans 2008, Bassolé and Juliani 2012). Bacteriostatic activity is seen as a reversible characteristic where, after an essential oil inhibits the growth of any specific bacteria, the microbe still has the capacity to recover reproductive capacity and some cells overcome the effects of the essential oil (Denyer

1995). On the other hand, bactericidal activity is measured by a permanent reduction and elimination of bacterial cells, where no cell once affected by an essential oil is capable of reproductive or growth actions (Denyer 1995). Though it is believed that the large number of components within essential oils obscure the specific microbial cellular targets of whole essential oils, research has speculated the detailed modes of action of various essential oil components (Carson, Mee et al. 2002, Hyldgaard, Mygind et al. 2012).

The broad-spectrum mode of action of essential oils is to act as lipophiles, damaging and disrupting the structure of a microorganisms' fatty acid based cytoplasmic membrane while passing through layers of polysaccharides, fatty acids and phospholipids to create gaps or pores, permeabilizing them (Bakkali, Averbeck et al. 2008). Bacterial membranes can also develop pores due to the reduction of ions, leading to a degeneration of proton channels and reduction of ATP energy pools (Di Pasqua, Betts et al. 2007). In addition to lipophilic activity, it is generally recognized that the phenolic, aldehyde and alcohol constituents of essential oils play a large role in bacterial membrane death (Bruni, Medici et al. 2004, Sacchetti, Maietti et al. 2005). Coagulation of the cytoplasm by essential oils and essential oil constituents can cause cell membranes to seep out macromolecules and ions or damage and leak proteins that result in cell death (apoptosis) (Cox, Gustafson et al. 1998, Burt 2004, Oussalah, Caillet et al. 2006). Research has suggested that the stage of bacterial growth influences the antibacterial efficacy of essential oils, i.e., essential oils can permeate budding sites of dividing cells more easily than established cells (Bakkali, Averbeck et al. 2008). It is also speculated that specific essential oils are more efficiently antimicrobial toward either Gram positive or Gram negative bacteria in relation to the pH of the oil (Faleiro 2011).

2.3.2. Antibacterial Research. The antibacterial activity of essential oils varies between the method of application, species of oil, concentrations of application, food product characteristics, synergistic combination of oils and transition from *in vitro* to *in situ* studies (Holley and Patel 2005, Bassolé and Juliani 2012, Hyldgaard, Mygind et al. 2012). Generally, the antibacterial activity of any particular essential oil is first evaluated based on Minimum Inhibitory Concentration (MIC) against strains of bacteria. (Škrinjar and Nemet 2009). Essential oils can then be added in dilutions to a bacterial broth and monitored for growth, called the broth dilution method. They can also be applied to a disk on a nutrient agar inoculated with bacteria and measured for a zone of inhibition, or area of non-growth around the application site, called the disc diffusion method (Singh 2011). Many studies using the above methods exist and are effective portrayals of essential oil potential as an antimicrobial, as shown in Table 2.1. After an essential oil has shown potential as an antibacterial, it is typically tested directly with a food product or packaging process and analyzed for influences on the product or package: practical inhibition or elimination of bacteria in the system at high or low concentrations, aroma, taste, texture, cost, simplicity, color changes, etc. A synopsis of recent studies applying essential oils to food products can be found in Table 2.2. While the examples in Table 2.2 are applied directly to the food product, studies shown in Table 2.3 are applied in an indirect method, meaning only the vapor or gaseous components of an essential oil were interacting with the microbial community on a food system. Vapor phase antimicrobial potency is unclear, though it has been theorized that since the lipophilic molecules of essential oils can associate to form micelles and suppress attachment to bacteria, the vapor phase form may allow for the free attachment of lipophiles and enhance inhibitory

activity *in vitro* (Laird and Phillips 2012). One example of an *in vitro* method is the microatmosphere method. Microatmosphere utilizes an inverted inoculated Petri dish. A small filter soaked with an essential oil is attached to the inside of the Petri dish top which, when the dish is turned upside down, is positioned directly under the inoculated medium so any vapor or gaseous components of the essential oil drift toward the bacteria (Ghalem and Mohamed 2008). Research and measurement methods of the vapor phase antimicrobial application of essential oils is still evolving as more information is obtained in synergism and food-oil interactions.

2.4. Modified Atmospheric Packaging

Modified Atmosphere Packaging (MAP) involves the manipulation of gases within a sealed vapor-barrier (McMillin, Huang et al. 1999). Three methods used for MAP are Vacuum Packaging (VP), standard MAP, and Controlled Atmosphere Packaging (CAP). Vacuum packaging compresses the air out of a partially packaged food product before heat sealing the package to prevent air refill. Standard MAP removes the current gases within a food package and replaces them with a new gas mixture, e.g. oxygen, carbon dioxide, carbon monoxide or nitrogen, before heat sealing the package with a headspace. Controlled Atmosphere Packaging constantly monitors and replaces the gaseous atmosphere of a food package to maintain a stable environment. The technology is used for time-sensitive ripening fruit which expel gases, e.g. ethylene and carbon dioxide, to trigger tissue softening (McMillin 2008). Currently, VP and MAP are occasionally utilized in RTE meat and food packaging according to quality and cost parameters (McMillin 2008).

2.4.1. Progression of MAP in retail meat packaging. The progression of grocery meat packaging, both RTE and fresh, originated with the wrapping of meat in waxed paper by butchers upon request. Packaging began to evolve upon the introduction of refrigerated retail meat display cases so customers could peruse and buy cuts without interaction with the butcher (Brody 2002). The need to openly store fresh and RTE meat for extended periods under constant light, withstanding safe handling by potential customers, and remaining transparent for clear viewing resulted in both lucent plastic overwrapping film for polystyrene tray packaging and more sturdy polymerized films for reduced moisture penetration (Kerry, O'grady et al. 2006). Primal cuts started to be reduced to smaller retail portions before shipping to the store, so VP became popular in shipping and storing to extend quality and shelf life, while increased food safety consciousness and the need to prolong the cherry-red color of raw beef or reddish-pink color of raw pork, which portrayed freshness, begot the manipulation of air within RTE and fresh meat packages (McMillin 2008).

The fresh color of raw meat is prolonged through the interaction of the myoglobin pigments and iron (Fe) in meat with oxygen; MAP extends the period of oxymyoglobin (Fe^{2+}) or carboxymyoglobin, the bright red or pink pigment associated with fresh meat, by increased exposure to oxygen (O_2) and small levels of carbon dioxide (CO_2) or carbon monoxide (CO) (Ščetar, Kurek et al. 2010, AMSA 2012). VP extends the period of deoxymyoglobin, the purple (Fe^{2+}) form of myoglobin, by removing oxygen and other gases to prevent the oxygenation of myoglobin until the package is opened and the meat reddens, or blooms, into the fresh color of oxymyoglobin (Ščetar, Kurek et al. 2010, AMSA 2012). Metmyoglobin is the browned or grayed form of the pigment (Fe^{3+}),

resulting from the oxidized pigments, and is associated with spoiled or old meat (McMillin 2008, Ščetar, Kurek et al. 2010, AMSA 2012). MAP is increasingly utilized in various sizes, gas concentrations, pouches and trays to extend the retail display time and quality of fresh and RTE meats because atmosphere and meat color is so closely entwined. The application of MAP for fresh and RTE meats has also opened the door to improve other aspects of meat quality such as color, taste, shelf-life, and moisture retention. Active packaging is described as the insertion of additives into packaged foods to extend quality and shelf-life, such as oxygen scavengers, moisture control compounds, carbon dioxide scavengers and emitters, and antimicrobial compounds or packaging designs (Ščetar, Kurek et al. 2010). The use of gaseous oxygen scavengers in atmospheric packaging is primarily researched to inhibit browning of meats. Carbon dioxide scavengers are researched to increase and hold the percentage of carbon dioxide in atmospheric packaging to lower the percentage of oxygen and create a partial vacuum, possibly inhibiting microbial growth and further discoloration of RTE and fresh meats in certain packaging. Moisture-absorbing compounds are studied to lower the amount of free moisture in packaged foods to discourage microbial growth and product spoilage. Moisture control can be achieved through the use of physical moisture-capture packaging techniques like pads and sachets, and oxygen and carbon dioxide scavenging compounds can be directly added into packaging and labels (Ščetar, Kurek et al. 2010). With leaps in food safety concerns, protocols, and microbial knowledge, antimicrobial packaging is becoming a widely sought application in RTE meat and food packaging.

2.4.2. Status of antimicrobial hurdles in RTE and fresh meat. Bacterial populations on fresh and RTE meats are primary causes of spoilage, e.g. odors, slime, discoloration and off-flavor, and foodborne illness (Butler, Bratzler et al. 1953). These populations are dependent on a large number of situations: health and handling of the live animal, contamination during slaughter, facility and plant employee sanitation, carcass chilling, fabrication sanitation, packaging, and storage temperature and time (McMillin 2008). Meat spoilage bacteria obtained from the above sources include: *Acinetobacter/Moraxella, Aeromonas, Alteromonas, Brochothrix thermosphacta, Lactobacillus, and Pseudomonas.* Pathogenic bacteria include: *Aeromonas hydrophila, Bacillus cereus, Campylobacter, Clostridium botulinum and perfringens, Escherichia coli 0157:H7, Listeria monocytogenes, Salmonella spp., and Staphylococcus aureus* (Kotula and Kotula 2000). Though improvements in sanitation, animal harvest, and food handling and preparation have led to reduced foodborne illness and general bacterial levels, contamination is unavoidable and food safety hurdles are required (Huffman 2002, Quintavalla and Vicini 2002). Current and researched food safety hurdles applied directly to food products which reduce or eliminate bacteria growth and survival include: heat processing, drying, freezing, refrigeration, irradiation, MAP, and adding antimicrobial compounds and salts (Quintavalla and Vicini 2002). However, fresh meat is not legally allowed to contain additives while RTE meat antimicrobials are bound by quality needs and consumer acceptability, so many hurdles cannot be applied (USDA-FSIS 2012). As such, one possible safety hurdle in the storage of fresh and RTE meat is the development of active packaging technology for MAP. The survival and growth rate of bacteria can be affected by MAP because spoilage bacteria are generally reduced in lowered oxygen

atmosphere. However, some pathogenic and spoilage bacteria develop levels of resistance to the fluctuating atmospheres between packaging and unpacking (Skandamis and Nychas 2002, McMillin 2008). For example, *Listeria monocytogenes*, a facultative anaerobic pathogen, has been shown to survive and grow despite the change between oxygen-rich and oxygen-poor food packaging and storage environments in the presence of growth medium and human food (Lungu, Ricke et al. 2009). Additional antimicrobial hurdles are constantly under research which can be utilized based on food quality and consumer acceptability needs such as: organic acids, acid salts, alcohols, fatty acids, bacteriocins, probiotics, enzymes, metals, antioxidants, antibiotics, fungicides, gases, sanitizers, and the volatiles and metabolites from plants and spices (Quintavalla and Vicini 2002, Cutter 2006, Zhou, Xu et al. 2010).

As evidenced by previous research presented in **Tables 2.1-2.3,** essential oils applied in gaseous and liquid form have shown promise in inhibition and elimination of pathogenic bacteria. Due to consumer trends opting for new natural alternatives to chemical safety hurdles, essential oil research has increased over the past few decades, particularly in RTE and fresh meat in MAP systems (Bassolé and Juliani 2012). The idea is to add small amounts of essential oil volatiles to the gas mixture used in present MAP systems and provide new food safety hurdles while presenting consumers with trending options perceived as "natural" and "safe". As discussed in previous sections, essential oil as antimicrobials still face barriers in RTE and fresh meat application due to synergism, plant origin, and organoleptic properties.

Table 2.1: Synopsis of literature testing individual and synergistic antibacterial activity of Essential Oils and Essential Oil constituents, in vitro

EOs or constituents	Common Names	Organisms	Concentrations	References
Carvacrol		*Listeria monocytogenes*	2, 2.5, 3 mmol^{-1}	(Karatzas, Kets et al. 2002)
Eucalyptus citriodora	Eucalyptus oil	*Escherichia coli*	10, 20, 30, 40, 50, 60, 70 µl/disc	(Nair, Vaghasiya et al. 2010)
		Staphylococcus aureus		
		Proteus mirabilis		
		Pseudomonas aeruginosa		
		Proteus vulgaris		
		Salmonella typhimurium		
		Enterobacter aerogenes		
		Pseudomonas testosterone		
		Alcaligenes fecalis		
		Bacillus cereus		
		Citrobacter freudii		
Coriandrum sativum	Coriander oil	*Escherichia coli*	MIC 0.1-1.6% v/v	(Silva, Ferreira et al. 2011)
		Klebsiella pneumonia		
		Salmonella typhimurium		
		Pseudomonas aeruginosa		
		Acinetobacter baumannii		
		Bacillus cereus		
		Staphylococcus aureus		
		Enterococcus faecalis		
Ocimum sanctum L.	Holy Basil	*Pseudomonas aeruginosa*	5,10 µl/mm	(Mahmood 2012)
		Escherichia coli		
		Klebsiella sp.		
		Proteus mirabulus		
		Staphylococcus aureus		
Pogostemon cablin	Patchouli	*Staphylococcus aureus*	MIC 0.156-5.0 µl/ml	(Lv, Liang et al. 2011)
Salvia sclarea	Clary Sage	*Bacillus subtilis*		
Rosmarinus officinalis	Rosemary	*Escherichia coli*		
Ocimum basilicum	Basil	*Saccharomyces cerevisiae*		
Mentha spicata	Spearmint			
Origanum vulgare	Oregano			
Perilla arguta	Perilla			
Artemisia absinthium	Absinthe			
Citrus bergamia	Bergamot			
Lavandula angustifolia	Lavender			
Allium sativum L./nisin	Garlic oil/nisin	*Listeria monocytogenes*	MIC 100µg/ml, 12.5IU/ml	(Razavi Rohani, Moradi et al. 2011)
Salviae aetheroleum	Sage oil	*Staphylococcus aureus*	MIC 0.097-0.390 mg/ml	(Bosnić, Softić et al. 2006)
Rosmarinus aetheroleum	Rosemary oil	*Bacillus subtilis*		
Eucalypti aetheroleum	Eucalyptus oil	*Escherichia coli*		
Melissae aetheroleum	Melissa oil	*Pseudomonas aeruginosa*		
Lavandulae aetheroleum	Lavender oil			
Thymus aetheroleum	Thyme oil			
Piper nigrum	Black pepper oil	*Acinetobacter calcoacetica*	15µl/disc	(Dorman and Deans 2008)
Syzygium aromaticum	Clove oil	*Aeromonas hydrophila*		
Pelargonium graveolens	Geranium oil	*Alcaligenes faecalis*		
Myristica fragrans	Nutmeg oil	*Bacillus subtilis*		
Origanum vulgare	Oregano oil	*Beneckea natriegens*		
Thymus vulgaris	Thyme oil	*Brevibacterium linens*		
		Brocothrix thermosphacta		
		Citrobacter freundii		
		Clostridium sporogenes		
		Enterococcus faecalis		
		Enterobacter aerogenes		
		Erwinia carotovora		
		Escherichia coli		
		Flavobacterium suaveolens		
		Klebsiella pneumonia		
		Lactobacillus plantarum		
		Leuconostoc cremoris		
		Micrococcus luteus		
		Moraxella sp.		
		Proteus vulgaris		
		Pseudomonas aeruginosa		
		Salmonella pullorum		
		Serratia marcescens		
		Staphylococcus aureus		
		Yersinia enterocolitica		
Ocimum basilicum	Basil oil	*Bacillus cereus*	50,100, 200 µl/ml	(Gutierrez, Barry-Ryan et al. 2008a)
Melissa officinalis	Lemon Balm oil	*Escherichia coli*		
Origanum majorana	Marjoram oil	*Listeria monocytogenes*		
Origanum vulgare	Oregano oil	*Pseudomonas aeruginosa*		
Rosmarinus officinalis	Rosemary oil			
Salvia triloba	Sage oil			
Thymus vulgaris	Thyme oil			
Lonicera japonica Thunb	Honeysuckle sp. oil	*Listeria monocytogenes*	MIC 62.5-500 µg/ml	(Rahman and Kang 2009)
		Bacillus subtilis		
		Bacillus cereus		
		Staphylococcus aureus		
		Salmonella enteritidis		
		Salmonella typhimurium		
		Enterobacter aerogenes		
		Escherichia coli		
		Pseudomonas aeruginosa		
Citrus bergamia	Bergamot oil	*Listeria monocytogenes*	5%-25% v/wt	(Ahmad, Benjakul et al. 2011)
Cymbopogon citratus	Lemongrass oil	*Pseudomonas aeruginosa*		
		Staphylococcus aureus		
		Salmonella typhimurium		

EO: Essential Oil, MIC: Minimum Inhibitory Concentration

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Table 2.2: Synopsis of Applied Food Product literature testing individual and synergistic antibacterial activity of Essential Oils and Essential Oil constituents, in situ

EOs or constituents	Common Names	Organisms	Food Product	Package/Process	Concentration	References
Carvacrol		*Listeria monocytogenes*	Semi-skimmed cow milk	High Pressure Processing	3 mmol^{-1}	(Karatzas, Kets et al. 2002)
Origanum vulgare	Oregano oil	Pseudomonads *Enterobacteriaceae*	Fresh chicken liver meat	Air permeable wrap, MAP	0.1% v/wt	(Hasapidou and Savvaidis 2011)
Eugenia caryophyllus	Clove oil	*Listeria monocytogenes*	Fresh minced mutton, Italian mozzarella cheese	Air permeable container	0.5% and 1% v/wt	(Vrinda Menon and Garg 2001)
Ally Isothiocyanate	Component of mustard, horseradish, wasabi oil	*Listeria monocytogenes* *Salmonella typhimurium*	Fresh Chicken breasts	MAP	0.3, 0.6, 1.2, 1.4, and 1.8 μg/h	(Shin, Harte et al. 2010)
Prunus armeniaca	Apricot oil	*Escherichia coli*	Apple juice	Air permeable container	10μl/v	(Friedman, Henika et al. 2004)
Citrus bergamia	Bergamot oil	*Salmonella enterica*				
Carvacrol						
Cinnamaldehyde						
Cinnamomum zeylanicum	Cinnamon Bark oil					
Cinnamomum cassia	Cinnamon Cassia oil					
Cinnamomum zeylanicum	Cinnamon Leaf oil					
Citral						
Syzygium aromaticum	Clove Bud oil					
Eugenol						
Geraniol						
Citrus paradisi	Grapefruit oil					
Lavandulae aetheroleum	Lavender oil					
Citrus x limon	Lemon oil					
Cymbopogon citratus	Lemongrass oil					
Citrus aurantifolia	Lime oil					
Linalool						
Linalyl acetate						
Melissae aetheroleum	Melissa oil					
Citrus aurantium	Orange bitter oil					
Citrus reticulata	Orange Mandarin oil					
Citrus sinensis	Orange sweet oil					
Origanum vulgare	Oregano oil, Spanish					
Citrus nobilis	Tangerine oil					
Terpinene						
Terpinen-4-ol						
Grape seed extract	ActiVin™	*Listeria monocytogenes*	Cooked Ground Beef	Air permeable plastic bag	1% v/wt	(Ahn, Grün et al. 2007)
Pine bark extract	Pycnogenol®	*Escherichia coli* 0157:H7				
Oleoresin Rosemary	Herbalox®	*Salmonella typhimurium* *Aeromonas hydrophila*				
Origanum vulgare	Oregano oil	*Enterobacteriaceae* *Pseudomonas* spp.	Fresh Beef	MAP, VP, air permeable		(Skandamis and Nychas 2002)
Thymus vulgaris	Thyme oil	*Escherichia coli*	Fresh Minced Beef	Air permeable bag	0.3%, 0.6%, 0.9% v/wt	(Solomakos, Govaris et al. 2008a, Solomakos, Govaris et al. 2008b)
Nisin		*Listeria monocytogenes*				
Origanum vulgare	Oregano oil	*Escherichia coli* 0157:H7	Fresh Feta Cheese	MAP	0.1ml/100g, 0.2ml/100g	(Govaris, Botsoglou et al. 2011)
Thymus vulgaris	Thyme oil	*Listeria monocytogenes*				
Rosmarinus officinalis	Rosemary oil	*Listeria monocytogenes*	RTE Fresh vegetables	Air permeable bag	1.25–5 μL/ml, 20–40 μL/mL	(De Azeredo, Stamford et al. 2011)
Origanum vulgare	Oregano oil	*Yersinia enterocolitica* *Aeromonas hydrophila* *Pseudomonas fluorescens*				
Origanum majorana	Marjoram oil	*Escherichia coli*	Fresh pork Sausage	Air permeable package	MIC 0.069-2.300 mg/mL	(Busatta, Vidal et al. 2008)

EO: Essential Oil, MIC: Minimum Inhibitory Concentration, MAP: Modified Atmosphere Packaging, VP: Vacuum Packaging

Table 2.3: Synopsis of literature testing the antibacterial activity of the vapor/gaseous phase of Essential Oils and Essential Oil constituents, in vitro and in situ

EOs or constituents	Common Names	Organisms	Food Product	Package/Process	Concentration	References
Coriandrum sativum *Foeniculum vulgare* *Anethum graveolens* *Mentha piperita* *Abies alba* *Juniperus communis* *Rosmarinus officinalis* *Lavandula hybrida*	Coriander oil Fennel oil Dill oil Peppermint oil Fir oil Juniper oil Rosemary oil Lavender oil	*Escherichia coli* *Staphylococcus aureus* *Candida albicans*		Microatmosphere	5-200µL/v	(ŞERBAN, IONESCU et al. 2011)
Eucalyptus globules *Eucalyptus camaldulensis*	Eucalyptus oil Eucalyptus oil	*Escherichia coli* *Staphylococcus aureus*		Microatmosphere	50µL/v	(Ghalem and Mohamed 2008)
Carvacrol Eugenol Menthol		*Escherichia coli* *Pseudomonas fluorescens* *Staphylococcus aureus* *Lactobacillus plantarum* *Bacillus subtilis* *Saccharomyces cerevisiae* *Botrytis cinerea*		Microatmosphere	0.1, 0.25, 0.5, 1, 2, 3 g l^{-1}	(Ben Arfa, Combes et al. 2006)
Armoracia rusticana	Horseradish root oil	*Enterobacteriaceae* *Pseudomonas* spp.	Cooked Roast Beef Slices	Sealed, Gas impermeable glass jar	20 µL	(Delaquis, Ward et al. 1999)
Cinnamomum zeylanicum *Thymus vulgaris* *Origanum vulgare* Cinnamaldehyde Thymol Carvacrol	Cinnamon oil Thyme oil Oregano oil	*Escherichia coli* *Yersinia enterocolitica* *Pseudomonas aeruginosa* *Salmonella choleraesuis* *Listeria monocytogenes* *Staphylococcus aureus* *Bacillus cereus* *Enterococcus faecalis* *Penicillium islandicum* *Aspergillus flavus* *Candida albicans*		Modified vapor diffusion test (Microatmosphere)	10µL	(López, Sánchez et al. 2007)
Allium sativum L. *Achillea millefolium L.* *Armoracia rusticana* *Artemisia absinthium* *Artemisia dracunculus* *Artemisia vulgaris* *Calamintha nepeta Savi* *Caryopteris x clandonensis* *Foeniculum vulgare* *Hypericum perforatum* *Hyssopus officinalis* *Lavandula angustifolia* *Mentha x piperita* *Mentha x villosa* *Nepeta grandiflora* *Nepeta x faassenii* *Ocimum basilicum* var. *grant vert* *Ocimum basilicum* var. *purple opal* *Origanum majorana* *Origanum vulgare* *Perovskia atriplicifolia* *Ruta Montana* *Salvia officinalis* *Satureja montana* *Thymus pulegioides* *Thymus serpyllum* *Thymus vulgaris*	Garlic oil Yarrow root Horseradish root Absinthe Wormwood Tarragon oil Common Wormwood Lesser Calamint Bluebeard Fennel oil St. John's Wort Hyssop oil True Lavender oil Peppermint oil Mentha villosa Giant Catmint Faassen's Catnip Basil variety Basil variety Marjoram Oregano Russian Sage oil Mountain Rue oil Common Sage oil Winter Savory oil Large Thyme oil Wild Thyme oil Thyme oil	*Escherichia coli* *Listeria monocytogenes* *Pseudomonas aeruginosa* *Salmonella enteritidis* *Staphylococcus aureus*		Microatmosphere	0.0083–0.53 µL/cm^3	(Nedorostova, Kloucek et al. 2009)
Origanum vulgare *Pimenta dioica* *Allium sativum L.*	Oregano oil Allspice plant extract Garlic oil	*Escherichia coli* O157:H7 *Listeria monocytogenes* *Salmonella enterica*	Tomato puree edible film	Vapor-phase diffusion (Microatmosphere)	0.5%, 1%, 1.5%, 3% w/w	(Du, Olsen et al. 2009)
Citrus limon *Citrus sinensis* *Citrus bergamia* Citral Linalool Limonene	Lemon oil Sweet Orange oil Bergamot oil	*Listeria monocytogenes* *Staphylococcus aureus* *Bacillus cereus* *Escherichia coli* O157:H7 *Campylobacter jejuni*	Cabbage leaves, chicken skin	Direct application and vapor diffusion	MIC 0.03-4% v/v	(Fisher and Phillips 2006)

EO: Essential Oil, MIC: Minimum Inhibitory Concentration

CHAPTER 3

MICROATMOSPHERE LISTERIA INHIBITION BY ESSENTIAL OILS

3.1. Summary

The purpose of this experiment was to assess the indirect inhibitory activity of volatile essential oils on *Listeria monocytogenes* (ATCC 4644) *in vitro* at three storage conditions (4°C for 10d, 24°C for 24h, 37°C for 24h). Four of six possible application volumes (10µL, 25µL, 50µL, 100µL, 150µL, 200µL) per oil were evaluated using a microatmosphere method and assessed visually for subjective inhibition. The essential oils or constituents applied in this study were: Spanish Basil oil (*Ocimum basilicum*), Bay oil (*Pimenta racemosa*), Italian Bergamot oil (*Citrus bergamia*), Roman Chamomile oil (*Anthemis nobilis*), Sri Lanka Cinnamon oil (*Cinnamomum zeylanicum*), Citral, Clove Bud oil (*Syzygium aromaticum*), Cumin Seed oil (*cuminum cyminum*), Eucalyptus oil (*Eucalyptus globulus*), Eugenol, Geranium extract (*Pelargonium graveolens*), Marjoram oil (*Origanum majorana*), Neroli extract (*Citrus aurantium*), Peppermint oil (*Mentha piperita* L.), Rosemary oil (*Rosmarinus officinalis* L.), and Spanish Sage oil (*Salvia officinalis* L.). Basil oil exhibited the least antilisterial activity across the three storage conditions, while peppermint, cumin seed, and citral showed the greatest inhibitory activity among all applications. Samples stored at 24°C for 24h, excepting chamomile and geranium, displayed enhanced inhibitory activity compared to similar samples stored at 4°C for 10d or 37°C for 24h. In conclusion, this experiment demonstrated varied levels of *in vitro* antilisterial inhibition by 16 essential oils and constituents at three storage

temperatures, supporting the indirect application of volatile essential oils as antimicrobials.

3.2. Introduction

A Ready-to-Eat (RTE) meat is a shelf-stable, refrigerated, or frozen product that has received a pathogen lethality treatment and does not require any other preparation before consumption, e.g. deli meats (Firouzi, Shekarforoush et al. 2007). Foodborne illnesses can result from these products when a lethality treatment fails or contamination occurs after the lethality treatment takes place. One possible contamination pathogen is *Listeria monocytogenes* (*LM*), known to survive on a variety of processing surfaces as well as subsist in both refrigeration (4℃) and variable room temperatures (24℃) (Thevenot, Dernburg et al. 2006). *LM* can result in the foodborne illness Listeriosis, a disease acutely dangerous to immunocompromised, pregnant, or elderly consumers because it can cause meningitis, febrile gastroenteritis, or septicemia (Ooi and Lorber 2005). The USDA instructs three methods of contamination prevention and control during the manufacturing of RTE meats: plant sanitation, antimicrobial ingredient formulation or packaging, and post-packaging lethality treatments. A large area of research exists within the application of antimicrobial ingredients and packaging, and the most commonly applied antilisterial ingredients today are sodium diacetate and sodium lactate (Zhu, Du et al. 2005). However, recent reports show that nearly half of polled shoppers disapproved of chemicals or additives in food, while approximately two-thirds of American consumers are concerned about the safety of their meat and poultry

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products. Products labeled as "natural" were viewed as more acceptable and likely to be considered healthy (Cava, Nowak et al. 2007, Berland 2012). Ingredient labeled with scientific names containing sodium, acids, or sugar derivatives were sometimes branded as having negative impacts on health (Berland 2012).

This perception of natural sounding ingredients and labels has expanded antimicrobial research of plant-based extracts and oils. Essential oils are the lipid-based secondary metabolites of plants which are distilled and used in a wide range of medicinal, fragrance, and food products. A variety of oils have been shown to possess antimicrobial activity against food pathogens, parasites, yeast and fungi, displayed in **Tables 2.1-2.3**. Because oils typically have strong flavor and aroma, the antimicrobial application in food products is unclear. Some oils, e.g., thyme and oregano, have displayed antimicrobial activity against pathogens when applied directly to foods such as minced beef or cooked chicken breasts (Solomakos, Govaris et al. 2008b, Govaris, Botsoglou et al. 2011). However, strong aroma or flavor can be off-putting to consumers. Indirect application of essential oils in gaseous or vapor dispersion could alleviate flavor intensity in food products, but may decrease the antimicrobial interaction between the oil and pathogen (Burt 2007).

The objective of this study was to quantify the indirect bacteriostatic ability of volatile essential oils on *L. monocytogenes in vitro* at different volumes and storage temperatures to determine if essential oil potency varies among food storage temperatures, as well as define the minimum volumes needed for listerial inhibition.

3.3. Materials and Methods

3.3.1. Materials and Sample Preparation

3.3.1.1. Essential Oils and Essential Oil Constituents. The essential oils and separate constituents used in this study are as follows: Spanish Basil oil (*Ocimum basilicum*), Bay oil (*Pimenta racemosa*), Italian Bergamot oil (*Citrus bergamia*), Roman Chamomile oil (*Anthemis nobilis*), Sri Lanka Cinnamon oil (*Cinnamomum zeylanicum*), Citral, Clove Bud oil (*Syzygium aromaticum*), Cumin Seed oil (*cuminum cyminum*), Eucalyptus oil (*Eucalyptus globulus*), Eugenol, Geranium extract (*Pelargonium graveolens*), Marjoram oil (*Origanum majorana*), Neroli extract (*Citrus aurantium*), Peppermint oil (*Mentha piperita* L.), Rosemary oil (*Rosmarinus officinalis* L.), and Spanish Sage oil (*Salvia officinalis* L.). Essential oils and separate constituents were purchased from Sigma-Aldrich (St. Louis, Missouri, United States). Oils and constituents were stored in sealed, light-sensitive brown glass bottles in a dark, ventilated, walk-in cooler at 5°C until needed.

While it has been discussed that essential oil component amounts vary by season, origin, and collection methods, previous scientific and fragrance corporation literature have determined general major and minor constituents for known essential oils (Omidbaigi, Sefidkon et al. 2004, Hussain, Anwar et al. 2008). **Table 3.1** provides a synopsis of commonly confirmed major constituents of the essential oils used in this experiment. The majority of constituents represented in **Table 3.1** are terpenoid in origin. For instance, Chamomile oil is known to contain sesquiterpene hydrocarbons as main constituents, while Marjoram oil yields mostly oxygen-containing and hydrocarbon

monoterpenes. Common monoterpenes present in **Table 3.1** include: linalool, pinene, 1,8-cineole, limonene, menthol, myrcene, and camphor. Eugenol, however, is an aromatic phenylpropanoid existing in large amounts in Clove oil, Cinnamon leaf oil, and Cumin Seed oil.

   3.3.1.2. Bacterial Strain and Culture Media. *Listeria monocytogenes* (ATCC 4644), was obtained from stock cultures stored on Brain Heart Infusion agar slants (BHI, Difco™ Laboratories, Sparks, MD, USA) at approximately 5°C and incubated in Brain Heart Infusion broth (BHI, Bacto™ Laboratories, Mt Pritchard, NSW, Australia) at 37°C for 48h. Modified Oxford Medium (MOX, Difco™ Laboratories, Sparks, MD, USA) agar was prepared in plastic Petri plates for use in the below microatmosphere trial. Selective MOX agar contains Difco™ antimicrobial supplement designed to hydrolyze esculin in response to *Listeria monocytogenes* growth, creating dark colored zones underneath the colonies. Selective MOX agar was chosen specifically due to the use of unsterile purchased essential oils and essential oil constituents.

3.3.2. Microatmosphere Method.

   The purpose of a microatmosphere method is to assess the indirect inhibitory effect of a compound on a bacterial strain without physical contact. The procedure used in this experiment was an adaptation of the microatmosphere method of vapor diffusion developed for essential oils (Ben Arfa, Combes et al. 2006, Ghalem and Mohamed 2008, ŞERBAN, IONESCU et al. 2011). Stock culture of *Listeria monocytogenes* (*LM*, ATCC 4644) was incubated in BHI broth at 37°C for 48h, adjusted to 0.1 abs (660nm absorbance) using a Bausch and Lomb Spectronic 70 Spectrophotometer (Irvine, CA,

USA), and applied to MOX agar Petri dishes with Eddy Jet™ spiral plater (IUL, Farmingdale, NY, USA) at an average inoculation of 6.18 Log_{10} CFU/ml. One autoclaved filter paper (Whatman® Grade 1, 1.5cm, Maidstone, UK) was adhered with double sided tape (Scotch Brand, Hutchinson, MN, USA) to the geographical center of each plastic Petri dish covering, suspended above the inoculated agar at approximately 1.27cm. Undiluted volumes of essential oils were chosen based on physical saturation capabilities of the filter paper. Maximum saturation was achieved at 200µl, so starting application volumes were determined as 50µl and 100µl to allow further one-half adjustments to a minimum of 10µl and 25µl and a maximum of 150µl and 200ul based on inhibition activity of the essential oil. Essential oils (0µl, 50µl, 100µl) were applied with sterile micropipettes to the filter papers of separate Petri dishes, in duplicate. Strips of 50.8mm Parafilm® M (Greenwich, CT, USA) were wrapped around each covered Petri dish, providing a partial sealant to prevent volatile seepage. Petri dishes were inverted, placed separately in closed glass jars to prevent further volatile atmospheric escape, and stored at either 24°C or 37°C for 24h or 4°C for 10 days before subjective visual evaluation for *LM* inhibition. After evaluation, the method was repeated for each essential oil at different volumes based on inhibition status: 10µl and 25µl volumes for complete inhibition or 150µl and 200µl for partial and no inhibition. A total of four volumes (50µl and 100µl in addition to 10µl and 25µl or 150µl and 200µl) at three temperatures (4°C, 24°C, and 37°C) were assessed per essential oil in this trial.

3.3.3. Measurement of Microatmosphere Activity

Determination of the minimal inhibition dose offered by the applied volumes of essential oils was measured on the presence or absence of growth, assessed visually. The minimal inhibition dose (MID) was defined as the minimal applied dose of essential oil required to completely inhibit bacteria growth for 24h at 37°C and 24°C and for 10 days at 4°C. Complete inhibition (CI) was viewed as a completely clear agar dish, no esculin color change, with no visible colony growth. Partial inhibition (PI) was judged as the presence of dark color zones and any visible colony growth less than that of inoculated control agar dishes with some clear or non-colonized zones suggesting a level of inhibition. No inhibition (NI) was observed as a complete lack of clear or unchanged agar zones, with no visible colonized differences from the inoculated, untreated control agar dishes.

3.4. Results and Discussion

3.4.1. Qualitative Microatmosphere Observation

Inhibition activity against *Listeria monocytogenes* varied among each essential oil when applied at three storage temperature levels. Basil oil displayed the least antilisterial activity across the three temperature applications. Peppermint, Cumin Seed, and Citral consistently exhibited the greatest antilisterial activity among the temperature applications. A Minimal Inhibitory Dose (MID) was obtained within the application volumes for essential oils stored at the three storage temperatures, depicted in **Tables 3.2-**

3.4, unless no complete inhibition (CI) was observed within the range of applied volumes, resulting in no declared MID.

Table **3.2** details the antilisterial inhibition status of the 16 essential oils stored at 37°C for 24 hours. Both Basil oil and Clove Bud oil were applied at 50µl, 100µl, 150µl, and 200µl, and no visual indication of inhibitory activity was detected at any volume, and no MID was declared for either oil. Basil oil is shown in **Figure 3.1**. Bay oil showed no inhibition at 50µl, but displayed partial inhibitory activity at 100µl, 150µl, and 200µl, seen in **Figure 3.2**, though no MID was assigned. Bergamot oil, however, achieved complete visual inhibition at 100µl, partial inhibition at 50µl, and no visual inhibition at 25µl or 10µl, so 100µl was declared as the MID. Chamomile oil and Eucalyptus oil both exhibited complete inhibition at 50µl (MID) and 100µl, with partial inhibition shown at 25µl and 10µl. Cinnamon oil and Neroli Extract displayed no inhibitory activity at 50µl or 100µl, but both showed partial inhibition at 150µl and 200µl (no MID). Citral, Cumin Seed oil, and Peppermint oil all achieved complete visual inhibition at 100µl, 50µl, 25µl, and 10µl (MID). Eugenol, however, displayed only partial inhibitory activity at 50µl, 100µl, 150µl, and 200µl (no MID). Geranium oil displayed inconsistent inhibition activity. Complete inhibition was achieved at 100µl, 50µl, and 10µl (MID), but narrowly incomplete/partial inhibition at 25µl. This result could have been due to application error or uneven dispersion due to an off-center filter paper. Marjoram oil showed complete inhibition at 100µl and 50µl (MID), partial inhibition at 25µl, and no inhibition at 10µl. Complete inhibition was achieved by Rosemary oil at both 50µl (MID) and 100µl, but no inhibition was observed at either 25µl or 10µl, seen in **Figure 3.3**. Lastly, Sage oil

displayed complete inhibitory activity at 100µl, 50µl, and 25µl (MID), with partial inhibition at 10µl.

Inhibition activity by the selected essential oils seemed to be somewhat related by common main constituents. Oils typically containing 1,8-cineole, for instance, all achieved complete inhibition of *Listeria monocytogenes* at 50µl or 100µl, sometimes at 10µl or 25µl. Eugenol-containing oils, such as Clove Bud, Bay, and Cinnamon oil, as well as Eugenol itself, all exhibited little to no inhibition activity at 150µl and 200µl. Oils that identified linalool as a major constituent in scientific literature displayed very different inhibition activity at any applied volume, ranging from the high inhibition activity of Cumin Seed and Geranium to the absence of inhibition activity by Basil oil; this suggests other main or minor constituents are beneficial. Many factors, such as vapor density, storage age, growth characteristics of *Listeria monocytogenes*, and constituent synergism could be attributed to varied results.

Table 3.3 details the inhibition status of the 16 essential oils stored at 24°C for 24 hours. Basil oil displayed no visual inhibitory activity at 50µl or 100µl, but partial inhibition at 150µl and 200µl (no MID), seen in **Figure 3.4**. Bay oil, Chamomile oil, Geranium oil, and Eugenol all displayed levels of partial inhibition from 50µl to 200µl (no MID), shown with Bay oil in **Figure 3.5**. Complete inhibition was exhibited by 8 oils from 100µl to 10µl (MID): Bergamot oil, Marjoram oil, Citral, Cumin Seed oil, Eucalyptus oil, Peppermint oil, Rosemary oil, and Sage oil. An example of complete inhibitory activity from 100µl to 10µl is shown with Rosemary oil in **Figure 3.6**. Cinnamon oil displayed partial inhibition from 50µl to 150µl, and achieved complete inhibition at 200µl (MID). Complete visual inhibition was observed by Clove Bud oil at

50µl (MID) and 100µl, while partial inhibition was seen at 25µl and 10µl. Lastly, Neroli Extract displayed complete inhibition from 100µl to 25µl (MID) and partial inhibition at 10µl.

Inhibition activity of the selected essential oils stored at 24°C were noticeably different in comparison to oils stored at 37°C for the same period of time. Eight oils displayed improved inhibition across all volumes: Basil oil, Bergamot oil, Cinnamon oil, Clove Bud oil, Marjoram, Neroli Extract, Rosemary oil, and Sage oil. Six were similar in activity at similar volumes: Bay oil, Citral, Cumin Seed oil, Eucalyptus, Eugenol, and Peppermint oil. Two oils, Chamomile and Geranium, exhibited limited inhibition activity at a higher range of volumes (50µl to 200µl) stored at 24°C compared to activity at a lower volume range (10µl to 100µl) at 37°C. One speculation is that the slightly slower growing time for *Listeria monocytogenes* allowed oil volatiles to more fully disrupt the organism at budding sites of the cells (Bakkali, Averbeck et al. 2008). Chamomile and Geranium oils, containing dissimilar main constituents than the other selected oils, could utilize a different mode of action or have active volatiles incompatible for room temperature bacterial inhibition.

Table 3.4 details the inhibition status of the 16 essential oils stored at 4°C for ten days. Basil oil exhibited no visually detectable inhibition from 50µl to 200µl (no MID), seen in **Figure 3.7**. Complete inhibition was observed for Bay oil at 200µl (MID), with partial inhibition at 150µl and no inhibition at 50µl and 100µl, shown in **Figure 3.8**. Bergamot oil displayed complete inhibition at 100µl (MID), while achieving partial inhibition at 50µl and 25µl and no inhibition at 10µl. Both Chamomile oil and Geranium oil showed no inhibition at 10µl and 25µl, but achieved partial inhibition at 50µl and

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complete inhibition at 100µl (MID). Cinnamon oil achieved complete inhibition at 150µl (MID) and 200µl, while showing no inhibition at 50µl and 100µl. Citral and Cumin Seed oil, however, exhibited complete inhibition from 10µl (MID) to 100µl. Clove Bud oil and Neroli Extract showed no inhibition at 50µl and 100µl, and achieved only partial inhibition at 150µl and 200µl (no MID). Complete inhibition was observed for Eucalyptus oil at 50µl (MID) and 100µl, partial inhibition at 25µl, and no inhibition at 10µl. Partial inhibition was observed for Eugenol from 50µl to 200µl (no MID). Marjoram oil exhibited complete inhibition from 25µl (MID) to 100µl, but no inhibition at 10µl. Complete inhibition was achieved by Peppermint oil at 50µl (MID) and 100µl, with partial inhibition at 25µl and 10µl. Both Rosemary oil and Sage oil displayed complete inhibition from 25µl (MID) to 100µl and partial inhibition at 10µl, seen with Rosemary oil in **Figure 3.8**.

The storage period for samples held at 4°C was extended to ten days due to the slow growth of *Listeria monocytogenes*, as indicated by control samples. The increased storage time and decreased temperature yielded enhanced inhibition activity for three oils, Clove Bud, Bay, and Cinnamon oil, when compared to samples held at 37°C for 24h. Chamomile, Geranium, and Bay oil improved inhibition activity when stored at 4°C for ten days in comparison to similar samples held at 24°C for 24h. Neroli oil and Basil oil displayed slightly lowered inhibition activity at 4°C than at 24°C.

Comparable studies which utilized the microatmosphere method reported many contrasting results, which could be expected in variable consideration of oil preparation, bacterial strain, and constituent disparity. Lopez et al, 2005, analyzed antilisterial inhibition with pure essential oil samples of Rosemary, Basil, Clove, and Cinnamon oil in

both direct application by disc diffusion and indirect application by microatmosphere at incubation storage temperature (Lopez, Sanchez et al. 2005). Rosemary and Basil were found to display some *Listeria monocytogenes* inhibition by direct application, but no inhibition activity using microatmosphere, whereas Rosemary oil achieved complete listerial inhibition with an application of 100µL at 37°C storage in this trial. Clove and Cinnamon oil, however, displayed a level of inhibition activity in the microatmosphere trial by Lopez et al only marginally weaker than direct application, while neither they nor Basil oil achieved a complete inhibition at 37°C in this trial. Goni et al, 2009, further explored the synergistic activity of Clove and Cinnamon oil, achieving partial inhibitions against *Listeria monocytogenes* in a microatmosphere study (Goñi, López et al. 2009). The authors speculated that the minor presence of 1,8-cineole and camphor in the essential oils enhanced the inhibition activity of eugenol, which was present in large amounts in both oils. A wide scope of synergistic research is possible using oil combinations containing those constituents, such as the combination of Rosemary and Bay oil. Nedorostova et al, 2009, completed a microatmosphere study somewhat methodologically similar to our trial, using an overlapping application volume of 32µL with a storage parameter of 37°C for 24 hours against *Listeria monocytogenes* (Nedorostova, Kloucek et al. 2009). Similar species of Peppermint, Basil, Marjoram, and Sage oil reported no listerial inhibition, in contrast to our results where only Basil failed to show inhibition activity at similar storage and application conditions. To our knowledge, few studies have evaluated the antilisterial inhibition activity of the selected essential oils near ambient or refrigeration temperatures, limiting result comparisons.

Most essential oils in this trial were assumed to impart antimicrobial activity through the accumulation of hydrophobic constituents, mostly monoterpenes, within the lipid portions of bacterial cell membrane structures, causing structural and functional damage. Both optimum ranges of hydrophobicity and the aqueous solubility of constituents could have contributed to varied inhibition activity (Goñi, López et al. 2009).

3.5. Conclusion

The selected essential oils, excepting Chamomile and Geranium, displayed some level of enhanced antilisterial inhibition activity when stored at 24°C for 24 hours, as compared to 37°C for 24 hours or 4°C for 10 days. The variation of inhibition activity by essential oils generally containing similar major constituents suggested a synergistic interaction of minor constituents to achieve inhibition. The inhibition variation of individual essential oils at the three storage temperatures could be attributed to vapor density, temperature-sensitive bacterial growth, the range of hydrophobicity within constituents, aqueous solubility of constituents, or unknown modes of action, though it was generally assumed the mode of action to be similar to that of lipophiles. Overall, this trial reported that various levels of inhibition activity could be achieved against *Listeria monocytogenes* at three storage temperatures.

Table 3.1: Major constituents of selected Essential Oils

Essential Oil	Major identified constituents	Primary References
Basil	Linalool, methyl chavicol	(Suppakul, Miltz et al. 2003, Hussain, Anwar et al. 2008)
Bay	Eugenol	(Höferl, Buchbauer et al. 2009)
Bergamot	Limonene, Linalyl acetate, linalool	(Verzera, Trozzi et al. 2003)
Chamomile	isobutyl angelate, 2-methyl butyl angelate, propyl tiglate, isoamyl angelate and 3-methylbutyl isobutyrate	(Omidbaigi, Sefidkon et al. 2004, Saroglou, Dorizas et al. 2006)
Cinnamon, leaf	Eugenol, linalool, pipertone	(Singh, Maurya et al. 2007)
Clove Bud	Eugenol, Benzyl alcohol, Eugenyl acetate	(Chaieb, Hajlaoui et al. 2007)
Cumin Seed	Cuminal, cuminic alcohol, γ-terpinene, safranal, ρ-cymene, β-pinene, 1,8-cineole, α-pinene, linalool	(Li and Jiang 2004, Iacobellis, Cantore et al. 2005)
Eucalyptus	1,8-cineole (eucalyptol)	(Edris 2007)
Geranium	Citronella, geraniol, linalool, isomethanone	(Babu and Kaul 2005)
Marjoram	1,8-cineole, limonene	(Miguel, Simoes et al. 2004)
Neroli	Linalool, β-pinene, α-terpineol, limonene, sabinene, nerol, nerolidol, linalyl acetate	(Wang, Zhu et al. 2012)
Peppermint	Menthol, 1,8-cineole, myrcene, α-pinene	(Ka, Choi et al. 2005, Yadegarinia, Gachkar et al. 2006)
Rosemary	Camphor, 1,8-cineole, α-pinene, linalool	(Carvalho, Moura et al. 2005, Uhart, Maks et al. 2006)
Sage	α-thujone, 1,8-cineole, camphor, borneol, β-pinene	(Longaray Delamare, Moschen-Pistorello et al. 2007)

Table 3.2: Inhibition status of selected Essential oils against L. monocytogenes at 37°C for 24h

Bacteria	*Listeria monocytogenes*					
Treatment	10 µl	25 µl	50 µl	100 µl	150 µl	200 µl
Basil oil			NI	NI	NI	NI
Bay oil			NI	PI	PI	PI
Bergamot oil	NI	NI	PI	CI^{MID}		
Chamomile oil	PI	PI	CI^{MID}	CI		
Cinnamon oil			NI	NI	PI	PI
Citral	CI^{MID}	CI	CI	CI		
Clove Bud oil			NI	NI	NI	NI
Cumin Seed oil	CI^{MID}	CI	CI	CI		
Eucalyptus oil	PI	PI	CI^{MID}	CI		
Eugenol			PI	PI	PI	PI
Geranium extract	CI^{MID}	PI	CI	CI		
Marjoram oil	NI	PI	CI^{MID}	CI		
Neroli extract			NI	NI	PI	PI
Peppermint oil	CI^{MID}	CI	CI	CI		
Rosemary oil	NI	NI	CI^{MID}	CI		
Sage oil	PI	CI^{MID}	CI	CI		

CI: Complete Inhibition, PI: Partial Inhibition, NI: No Inhibition,
MID: Minimal Inhibitory Dose

Table 3.3: Inhibition Status of selected Essential Oils against L. monocytogenes at 24°C for 24h

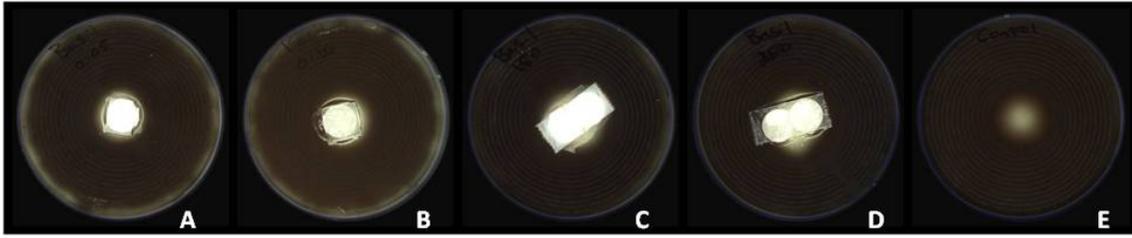| Bacteria	*Listeria monocytogenes*					
Treatment	10 µl	25 µl	50 µl	100 µl	150 µl	200 µl
Basil oil			NI	NI	PI	PI
Bay oil			PI	PI	PI	PI
Bergamot oil	CI^{MID}	CI	CI	CI		
Chamomile oil			PI	PI	PI	PI
Cinnamon oil			PI	PI	PI	CI^{MID}
Citral	CI^{MID}	CI	CI	CI		
Clove Bud oil	PI	PI	CI^{MID}	CI		
Cumin Seed oil	CI^{MID}	CI	CI	CI		
Eucalyptus oil	CI^{MID}	CI	CI	CI		
Eugenol			PI	PI	PI	PI
Geranium extract			PI	PI	PI	PI
Marjoram oil	CI^{MID}	CI	CI	CI		
Neroli extract	PI	CI^{MID}	CI	CI		
Peppermint oil	CI^{MID}	CI	CI	CI		
Rosemary oil	CI^{MID}	CI	CI	CI		
Sage oil	CI^{MID}	CI	CI	CI		

CI: Complete Inhibition, PI: Partial Inhibition, NI: No Inhibition
MID: Minimal Inhibitory Dose

Table 3.4: Inhibition Status of selected Essential Oils against L. monocytogenes at 4°C for 10 days

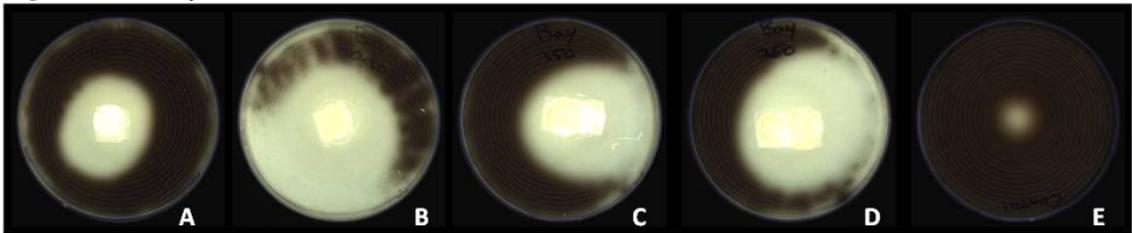| Bacteria	*Listeria monocytogenes*					
Treatment	10 µl	25 µl	50 µl	100 µl	150 µl	200 µl
Basil oil			NI	NI	NI	NI
Bay oil			NI	NI	PI	CI^{MID}
Bergamot oil	NI	PI	PI	CI^{MID}		
Chamomile oil	NI	NI	PI	CI^{MID}		
Cinnamon oil			NI	NI	CI^{MID}	CI
Citral	CI^{MID}	CI	CI	CI		
Clove Bud oil			NI	NI	PI	PI
Cumin Seed oil	CI^{MID}	CI	CI	CI		
Eucalyptus oil	NI	PI	CI^{MID}	CI		
Eugenol			PI	PI	PI	PI
Geranium extract	NI	NI	PI	CI^{MID}		
Marjoram oil	NI	CI^{MID}	CI	CI		
Neroli extract			NI	NI	PI	PI
Peppermint oil	PI	PI	CI^{MID}	CI		
Rosemary oil	PI	CI^{MID}	CI	CI		
Sage oil	PI	CI^{MID}	CI	CI		

CI: Complete Inhibition, PI: Partial Inhibition, NI: No Inhibition
MID: Minimal Inhibitory Dose

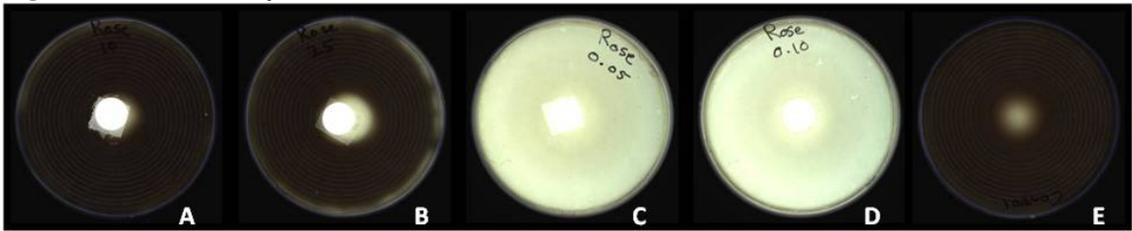**Figure 3.1**: Basil Oil stored at 37°C for 24h

Basil Oil was applied to the inside lid of a *Listeria monocytogenes* inoculated Modified Oxford Petri agar in the following volumes: (A) 50µL, (B) 100µL, (C) 150µL, (D) 200µL, (E) Control/No Application. Levels of *Listeria monocytogenes* inhibition are labeled as: (A) No Inhibition, (B) No Inhibition, (C) No Inhibition, (D) No Inhibition, (E) No Inhibition. Complete inhibition (CI) was viewed as a completely clear agar dish, no esculin color change, with no visible colony growth. Partial inhibition (PI) was judged as the presence of dark color zones and any visible colony growth less than that of inoculated control agar dishes with some clear or non-colonized zones suggesting a level of inhibition. No inhibition (NI) was observed as a complete lack of clear or unchanged agar zones underneath colonies, with no visible colonized differences from the inoculated, untreated control agar dishes.
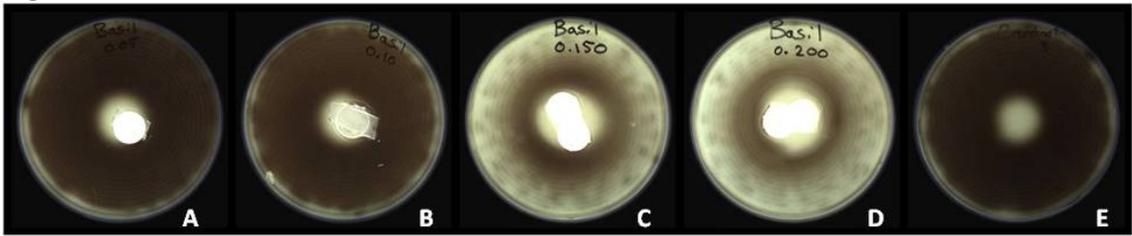
Figure 3.2: Bay Oil stored at 37°C for 24h

Bay Oil was applied to the inside lid of a *Listeria monocytogenes* inoculated Modified Oxford Petri agar in the following volumes: (A) 50µL, (B) 100µL, (C) 150µL, (D) 200µL, (E) Control/No Application. Levels of *Listeria monocytogenes* inhibition are labeled as: (A) Partial Inhibition, (B) Partial Inhibition, (C) Partial Inhibition, (D) Partial Inhibition, (E) No Inhibition. Complete inhibition (CI) was viewed as a completely clear agar dish, no esculin color change, with no visible colony growth. Partial inhibition (PI) was judged as the presence of dark color zones and any visible colony growth less than that of inoculated control agar dishes with some clear or non-colonized zones suggesting a level of inhibition. No inhibition (NI) was observed as a complete lack of clear or unchanged agar zones underneath colonies, with no visible colonized differences from the inoculated, untreated control agar dishes.

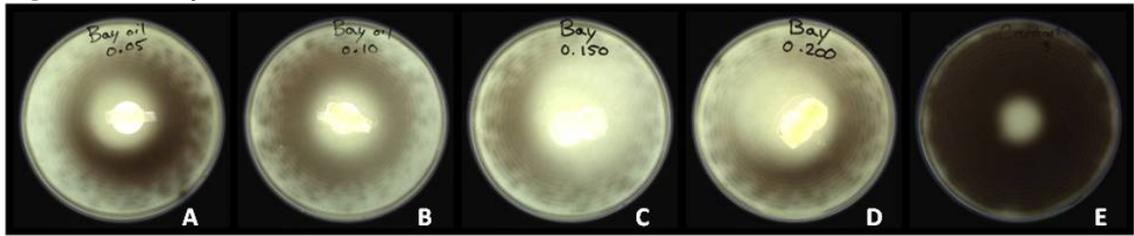**Figure 3.3**: Rosemary Oil stored at 37°C for 24h

Rosemary Oil was applied to the inside lid of a *Listeria monocytogenes* inoculated Modified Oxford Petri agar in the following volumes: (A) 10µL, (B) 25µL, (C) 50µL, (D) 100µL, (E) Control/No Application. Levels of *Listeria monocytogenes* inhibition are labeled as: (A) No Inhibition, (B) No Inhibition, (C) Complete Inhibition, (D) Complete Inhibition, (E) No Inhibition. Complete inhibition (CI) was viewed as a completely clear agar dish, no esculin color change, with no visible colony growth. Partial inhibition (PI) was judged as the presence of dark color zones and any visible colony growth less than that of inoculated control agar dishes with some clear or non-colonized zones suggesting a level of inhibition. No inhibition (NI) was observed as a complete lack of clear or unchanged agar zones underneath colonies, with no visible colonized differences from the inoculated, untreated control agar dishes.

Figure 3.4: Basil Oil stored at 24°C for 24h

Basil Oil was applied to the inside lid of a *Listeria monocytogenes* inoculated Modified Oxford Petri agar in the following volumes: (A) 50µL, (B) 100µL, (C) 150µL, (D) 200µL, (E) Control/No Application. Levels of *Listeria monocytogenes* inhibition are labeled as: (A) No Inhibition, (B) No Inhibition, (C) Partial Inhibition, (D) Partial Inhibition, (E) No Inhibition. Complete inhibition (CI) was viewed as a completely clear agar dish, no esculin color change, with no visible colony growth. Partial inhibition (PI) was judged as the presence of dark color zones and any visible colony growth less than that of inoculated control agar dishes with some clear or non-colonized zones suggesting a level of inhibition. No inhibition (NI) was observed as a complete lack of clear or unchanged agar zones underneath colonies, with no visible colonized differences from the inoculated, untreated control agar dishes.
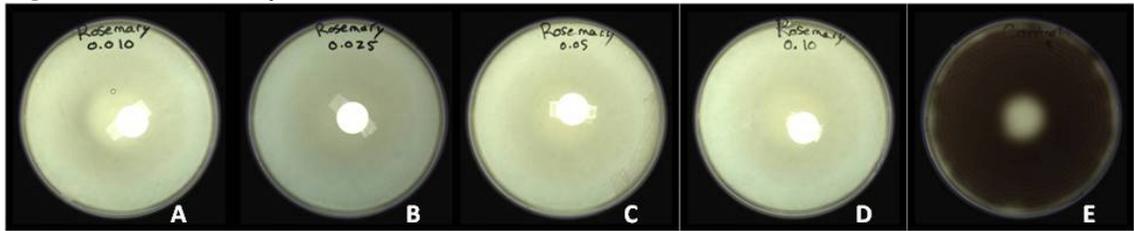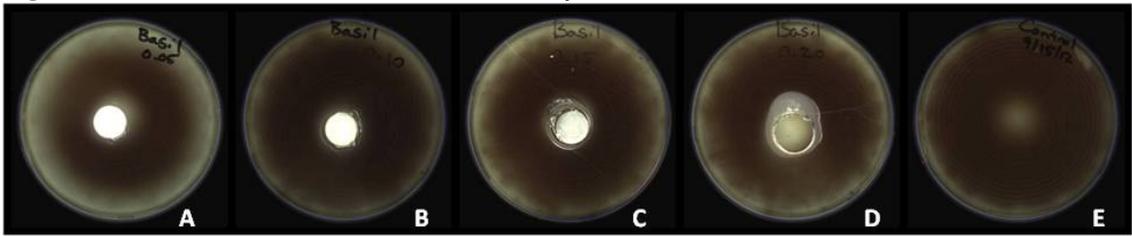
Figure 3.5: Bay Oil stored at 24°C for 24h

Bay Oil was applied to the inside lid of a *Listeria monocytogenes* inoculated Modified Oxford Petri agar in the following volumes: (A) 50µL, (B) 100µL, (C) 150µL, (D) 200µL, (E) Control/No Application. Levels of *Listeria monocytogenes* inhibition are labeled as: (A) Partial Inhibition, (B) Partial Inhibition, (C) Partial Inhibition, (D) Partial Inhibition, (E) No Inhibition. Complete inhibition (CI) was viewed as a completely clear agar dish, no esculin color change, with no visible colony growth. Partial inhibition (PI) was judged as the presence of dark color zones and any visible colony growth less than that of inoculated control agar dishes with some clear or non-colonized zones suggesting a level of inhibition. No inhibition (NI) was observed as a complete lack of clear or unchanged agar zones underneath colonies, with no visible colonized differences from the inoculated, untreated control agar dishes.

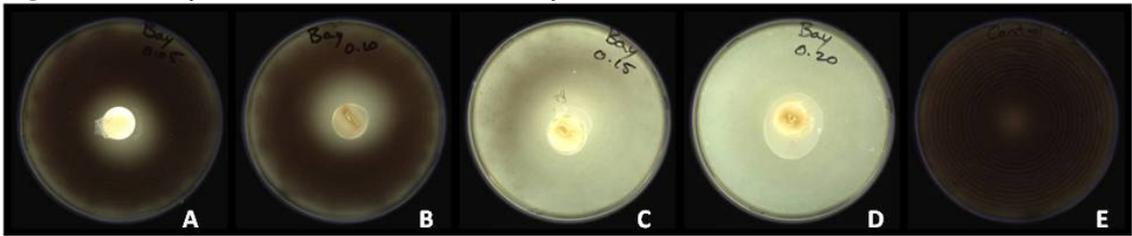**Figure 3.6**: Rosemary Oil stored at 24°C for 24h

Rosemary Oil was applied to the inside lid of a *Listeria monocytogenes* inoculated Modified Oxford Petri agar in the following volumes: (A) 10µL, (B) 25µL, (C) 50µL, (D) 100µL, (E) Control/No Application. Levels of *Listeria monocytogenes* inhibition are labeled as: (A) Complete Inhibition, (B) Complete Inhibition, (C) Complete Inhibition, (D) Complete Inhibition, (E) No Inhibition. Complete inhibition (CI) was viewed as a completely clear agar dish, no esculin color change, with no visible colony growth. Partial inhibition (PI) was judged as the presence of dark color zones and any visible colony growth less than that of inoculated control agar dishes with some clear or non-colonized zones suggesting a level of inhibition. No inhibition (NI) was observed as a complete lack of clear or unchanged agar zones underneath colonies, with no visible colonized differences from the inoculated, untreated control agar dishes.

Figure 3.7: Basil Oil stored at 4°C for 10 days

Basil Oil was applied to the inside lid of a *Listeria monocytogenes* inoculated Modified Oxford Petri agar in the following volumes: (A) 50µL, (B) 100µL, (C) 150µL, (D) 200µL, (E) Control/No Application. Levels of *Listeria monocytogenes* inhibition are labeled as: (A) No Inhibition, (B) No Inhibition, (C) No Inhibition, (D) No Inhibition, (E) No Inhibition. Complete inhibition (CI) was viewed as a completely clear agar dish, no esculin color change, with no visible colony growth. Partial inhibition (PI) was judged as the presence of dark color zones and any visible colony growth less than that of inoculated control agar dishes with some clear or non-colonized zones suggesting a level of inhibition. No inhibition (NI) was observed as a complete lack of clear or unchanged agar zones underneath colonies, with no visible colonized differences from the inoculated, untreated control agar dishes.

Figure 3.8: Bay Oil stored at 4°C for 10 days

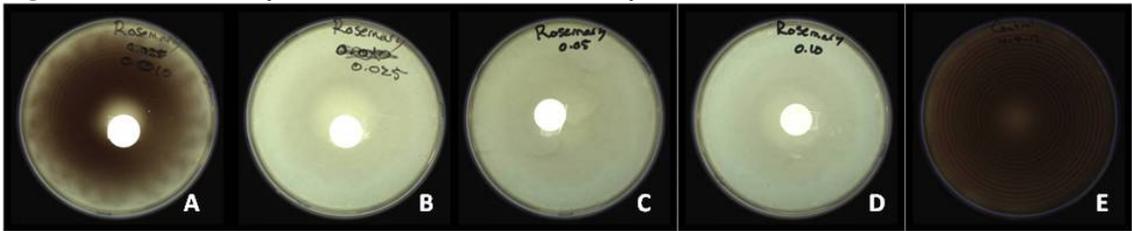

Bay Oil was applied to the inside lid of a *Listeria monocytogenes* inoculated Modified Oxford Petri agar in the following volumes: (A) 50µL, (B) 100µL, (C) 150µL, (D) 200µL, (E) Control/No Application. Levels of *Listeria monocytogenes* inhibition are labeled as: (A) No Inhibition, (B) No Inhibition, (C) Partial Inhibition, (D) Complete Inhibition, (E) No Inhibition. Complete inhibition (CI) was viewed as a completely clear agar dish, no esculin color change, with no visible colony growth. Partial inhibition (PI) was judged as the presence of dark color zones and any visible colony growth less than that of inoculated control agar dishes with some clear or non-colonized zones suggesting a level of inhibition. No inhibition (NI) was observed as a complete lack of clear or unchanged agar zones, with no visible colonized differences from the inoculated, untreated control agar dishes.

Figure 3.9: Rosemary Oil stored at 4°C for 10 days

Rosemary Oil was applied to the inside lid of a *Listeria monocytogenes* inoculated Modified Oxford Petri agar in the following volumes: (A) 10µL, (B) 25µL, (C) 50µL, (D) 100µL, (E) Control/No Application. Levels of *Listeria monocytogenes* inhibition are labeled as: (A) Partial Inhibition, (B) Complete Inhibition, (C) Complete Inhibition, (D) Complete Inhibition, (E) No Inhibition. Complete inhibition (CI) was viewed as a completely clear agar dish, no esculin color change, with no visible colony growth. Partial inhibition (PI) was judged as the presence of dark color zones and any visible colony growth less than that of inoculated control agar dishes with some clear or non-colonized zones suggesting a level of inhibition. No inhibition (NI) was observed as a complete lack of clear or unchanged agar zones, with no visible colonized differences from the inoculated, untreated control agar dishes.

CHAPTER 4

LISTERIAL INHIBITION BY ESSENTIAL OILS IN A MAP SYSTEM

4.1. Summary

The purpose of this trial was to observe the antilisterial (*Listeria monocytogenes*, ATCC 4644) activity of volatile essential oils when applied adjacent to, but not touching, bologna slices within a Modified Atmosphere Packaging (MAP, 80% O_2, 20% CO_2) system. The 16 essential oils or constituents applied in this study have displayed previous antibacterial activity: Spanish Basil oil (*Ocimum basilicum*), Bay oil (*Pimenta racemosa*), Italian Bergamot oil (*Citrus bergamia*), Roman Chamomile oil (*Anthemis nobilis*), Sri Lanka Cinnamon oil (*Cinnamomum zeylanicum*), Citral, Clove Bud oil (*Syzygium aromaticum*), Cumin Seed oil (*cuminum cyminum*), Eucalyptus oil (*Eucalyptus globulus*), Eugenol, Geranium extract (*Pelargonium graveolens*), Marjoram oil (*Origanum majorana*), Neroli extract (*Citrus aurantium*), Peppermint oil (*Mentha piperita* L.), Rosemary oil (*Rosmarinus officinalis* L.), and Spanish Sage oil (*Salvia officinalis* L.). Eugenol applied at 1.35ml resulted in a mean of one $\log_{10}$ CFU/ml reduction in *LM*, but could not be replicated. Peppermint oil ($P< 0.048$) displayed significant differences between applied volumes (0.13ml and 1.35ml) but did not reach a one $\log_{10}$ CFU/ml reduction of *LM*. No other oils displayed any significant inhibitory activity against *LM*. This trial suggests that whole oils which have displayed inhibitory characteristics against *in vitro* may need further study of modes of action or application volumes to transition into a food system.

4.2. Introduction

Strides have been made to embrace essential oils and essential oil constituents as antimicrobials through research, as seen in **Tables 2.1-2.3**. Incorporation into a food system is generally the next step once an oil or constituent is identified as a possible antimicrobial *in vitro*. However, studies have shown that antimicrobial potency can change in food systems due to environmental factors such as packaging, bacterial growth characteristics, and food-oil interaction (Burt 2007, Dorman and Deans 2008). In addition, essential oils and constituents tend to possess strong organoleptic characteristics, which can decrease the consumer appeal of many foods. One method to lessen the organoleptic effects of essential oils in a food system is to use an indirect application, such as vapor interaction. The use of essential oil vapors as antimicrobials is gaining attention, though research is still varied and limited on the subject (Laird and Phillips 2012).

The application of antimicrobial vapor is attractive to Modified Atmosphere Packaging (MAP) since the incorporation of gas is already present in these processes. MAP involves the manipulation of gases, typically oxygen and carbon dioxide, within a sealed vapor-impermeable package (McMillin 2008). Active packaging includes the addition of antimicrobials to a food packaging system, and research and industry applications have increased in the past decades (Ščetar, Kurek et al. 2010). Adding essential oil vapor to MAP food systems is a quite new area of research. The mode of action by which essential oil vapors inhibit various bacteria in comparison to physical contact is unclear, and the potency against selected bacteria changes between direct and

indirect contact (Laird and Phillips 2012). For this reason, increased experimental research is needed to explore the effects of essential oil volatiles with food systems. The objective of this research was to observe the indirect *in situ* antimicrobial effects of volatile essential oils within a MAP food system.

4.3. Material and Methods

4.3.1. Materials and Sample Preparation

Bologna used in this study was prepared at the University of Kentucky Meat Laboratory (Lexington, KY). Pork retail cuts of Boston Butt and Picnic Shoulder were deboned and emulsified (Mainca CM-14 22lb capacity Bowl Chopper, St. Louis, MO, USA). Legg's Old Plantation Seasoning (Calera, AL, USA) containing 6.25% sodium nitrite per 1 oz was incorporated during the emulsification; no *L. monocytogenes* inhibition compounds such as sodium lactate and sodium diacetate were included. Meat batter was hand-crank filled into inedible collagen bologna casing (Koch, 150mm x 470mm, Kansas City, MO, USA) and cooked to an approximate internal temperature of 71°C (Enviro-Pak Red Arrow Powersmoker 100, Clackamas, OR, USA). Upon cooling bologna was sliced (Hobart Industrial Slicer, Troy, OH, USA) to a thickness of 6.35mm, placed in vacuum sealed storage bags and stored in a -20°C freezer until needed. Final bologna formulation was 58.17% moisture, 25.71% fat, and 8.7% salt with a pH of 6.29.

4.3.1.1. Essential Oils and Essential Oil Constituents. The essential oils and separate constituents used in this study are as follows: Spanish Basil oil (*Ocimum basilicum*), Bay oil (*Pimenta racemosa*), Italian Bergamot oil (*Citrus bergamia*), Roman Chamomile oil (*Anthemis nobilis*), Sri Lanka Cinnamon oil (*Cinnamomum zeylanicum*), Citral, Clove Bud oil (*Syzygium aromaticum*), Cumin Seed oil (*cuminum cyminum*), Eucalyptus oil (*Eucalyptus globulus*), Eugenol, Geranium extract (*Pelargonium graveolens*), Marjoram oil (*Origanum majorana*), Neroli extract (*Citrus aurantium*), Peppermint oil (*Mentha piperita* L.), Rosemary oil (*Rosmarinus officinalis* L.), and Spanish Sage oil (*Salvia officinalis* L.). Essential oils and separate constituents were purchased from Sigma-Aldrich (St. Louis, Missouri, United States). Oils and constituents were stored in sealed, light-sensitive brown glass bottles in a dark, ventilated, walk-in cooler at 5°C until needed. Volumes applied in this study were determined by the minimal inhibition dose (MID) and highest inhibition dose presented in Chapter 3.

4.3.1.2. Bacterial Strain and Culture Media. The bacteria applied for this research, *Listeria monocytogenes* (ATCC 4644), was obtained from stock cultures stored on Brain Heart Infusion agar slants (BHI, Difco™ Laboratories, Sparks, MD, USA) at approximately 5°C and grown in Brain Heart Infusion broth (BHI, Bacto™ Laboratories, Mt Pritchard, NSW, Australia) at 37°C incubation in two day advancements as needed. Modified Oxford Medium (MOX, Difco™ Laboratories, Sparks, MD, USA) agar was prepared in plastic Petri plates for use in measurement of colony counts after inoculated bologna sample collection and dilution. Selective MOX agar contains Difco™ antimicrobial supplement designed to hydrolyze esculin in response to *Listeria monocytogenes* growth, creating dark colored zones underneath the colonies. Selective

MOX agar was utilized in this trial due to the application of unsterile purchased essential oils and feasible contamination within bologna formulation and slicing.

4.3.1.3. Essential Oil Dose Volume Conversion. Essential oil volumes used in this MAP trial were determined using Chapter 3 data. Water displacement comparisons were made of the total air volume between the plastic Petri dishes used in Chapter 3 (77.5ml) and the plastic MAP trays used in this trial (1045.5ml), and a scale was developed to adjust Chapter 3 inhibition volumes to the larger MAP trays. The volume conversions from Chapter 3 are presented in Table 4.1.

4.3.2. Modified Atmosphere Packaging (MAP)

Stored, previously frozen bologna slices were thawed for 12h and partitioned to individual plastic MAP trays (4.32cm)**.** Stock culture of *Listeria monocytogenes* (*LM*, ATCC 4644) was incubated in BHI broth at 37°C for 48h , adjusted to 0.1 abs (660nm absorbance) using a Bausch and Lomb Spectronic 70 Spectrophotometer (Irvine, CA, USA), and applied to individual bologna slices at 100µl with a sterile micropipette. Inoculum was spread evenly over the bologna surface with a sterile plastic spread stick for an average inoculation of 7.9 $\log_{10}$ colony-forming units per ml (CFU/ml). One sterile filter paper (7.0cm. Whatman Grade 1, Maidstone, UK) was uniformly adhered inside each MAP tray with double sided tape (Scotch Brand, Hutchinson, MN, USA), adjacent to but not touching the inoculated bologna slice. All samples were then packaged in 80% O_2, 20% CO_2 mixed gas atmosphere with a Ross InPack Junior A10 MAP (Midland, VA, USA) machine. Individual rubber adhesive seals were attached to the sealed film of each packaged tray, directly above the inner filter paper. Essential oil volumes of 0.13ml,

1.35ml, or 2.70ml were injected through the rubber seals using sterile 1cc or 3cc medical needle syringes directly onto the filter paper. Samples were stored at 24°C for 24h. A total of 16 oils were applied in this trial at 2 volumes each (0.13ml and 1.35ml or 1.35ml and 2.70ml) with duplicates of both untreated inoculated and untreated non-inoculated bologna sample control packages.

4.3.3. Measurement of MAP Trial

After 24h storage at 24°C, samples were aseptically removed from packaging and placed in plastic stomacher bags. One percent Peptone dilution solution (Bacto™ Laboratories, Mt Pritchard, NSW, Australia) was prepared and autoclaved. Peptone solution was added to whole sample bags at one-third dilutions based on individual sample weight, and samples were agitated for approximately one minute in a Stomacher Lab-Blender 400 (Worthing, West Sussex, UK). Liquid from each sample was then collected and adjusted in pH neutral solution to a one-three thousandth dilution and plated in duplicate on MOX agar using an EddyJet™ Spiral Plater (IUL, Farmingdale, NY, USA). Agar plates were incubated at 37°C for 24h, and colony growth was measured in Log_{10} Colony Forming Units (CFU/ml) using a Flash & Go™ Plate Reader (IUL, Farmingdale, NY, USA).

4.3.4. Statistical Analysis

Significant differences in the Percent Change (% Change) between the two volumes of each essential oil were determined by independent two-tailed Student's *t* test at the 95% significance level using SAS (SAS Institute Inc., Cary, NC, USA). The %

Change was defined as the percentage of growth (+ value) or inhibition (- value) compared to the control inoculated sample without an oil application (*((sample growth – control growth)/control growth) x 100*). Every ten percent change represented approximately one Log_{10} CFU/ml difference from the control samples, which was determined as a practical reduction in *LM*.

4.4. Results and Discussion

4.4.1. MAP Trial

Un-inoculated control samples in this trial consistently measured zero *LM* growth, while inoculated control samples in this trial consistently measured an average of 7.73 Log_{10} CFU/ml *LM* growth. Peppermint oil ($P< 0.048$) resulted in a statistically significant (95% significance level) reduction in *LM* between 0.13ml and 1.35ml samples seen in **Table 4.2**. However, Peppermint oil achieved less than one log reduction in both sample means, resulting in no practical reduction in *LM*. Only Eugenol (1.35ml, 10.181% Change (-)) exhibited a mean one log reduction in *LM*, but the reduction could not be replicated. Clove Bud oil (0.13ml, 16.31% Change (+)) and Neroli extract (0.13ml, 9.22 % Change (+)) actually exhibited increased LM growth compared to control samples. The activity of Eugenol, Clove Bud oil, and Neroli extract are anomalies in this trial, and could be attributed to method error. **Figure 4.1** shows the *LM* colony counts collected from bologna samples treated with either 1.35ml or 2.70ml Basil oil, of which linalool is typically the main constituent. Chapter 3 showed Basil oil achieved partial inhibition of *LM* at both 150µl and 200µl when stored at 24°C for 24h. However, when scaled to the

equivalent volume in this trial (2.70ml) no antilisterial activity was detected at the same storage temperature and time. Colony counts collected from bologna samples treated with Eugenol (1.35ml or 2.70ml) are shown in **Figure 4.2**, and result in the same performance comparison as Basil oil. Eucalyptus oil and Rosemary oil (10µl) were reported in Chapter 3 to both achieve complete inhibition against LM at 24°C for 24h, the common main constituent being 1,8-cineole. However, colony counts collected from bologna samples treated with 0.13ml or 1.35ml of either oil in this trial, **Figure 4.3 and 4.4**, all displayed less than one log reduction in *LM* when stored at 24°C for 24h. Less than one log reduction was observed by 15 of 16 essential oils in this trial, excepting Eugenol, in contrast to the 11 essential oils which achieved complete inhibition at any volume when stored at 24°C for 24 hours, shown in **Table 3.3**.

Few studies are directly comparable to this experiment by a combination of essential oil and sample use. However, Fisher et al (2006) tested the vapor forms of bergamot, citral, and linalool against bacterial strains that included *Listeria monocytogenes* on cabbage leaves for 8-10 hours of exposure (Fisher and Phillips 2006). In that case, the whole oil and two oil constituents displayed an average of 6 Log_{10} CFU/ml reductions of LM, which is dramatically contrasting to the detailed experiment. This experiment saw no reduction of LM in response to exposure of bergamot, citral, or basil oil of which linalool is the major constituent.

The translation from a broth or agar application to an inhibitory activity in a food product has generally shown to decrease the efficacy of essential oils, regardless of indirect or direct application (Burt 2004). For instance, nutmeg and oregano oils were effective against *E.coli* 0157:H7 in broth medium, but ineffective when applied to raw

chicken (Firouzi, Shekarforoush et al. 2007). Spices which displayed inhibition activity against *Salmonella* directly also decreased in antimicrobial effect when applied to ground beef (Uhart, Maks et al. 2006). One attribute of decreased inhibitory activity could be the protein and fat associated with the food product, as well as the pH of the food. High concentrations of meat proteins enhanced the growth characteristics of *Listeria monocytogenes* (Gutierrez, Barry-Ryan et al. 2008a). Higher amounts of animal and plant based fats in comparison to reduced fat samples have shown to decrease the antimicrobial activity of essential oils in milk, hot dogs, and beef (Singh, Singh et al. 2003, Cava, Nowak et al. 2007, Gutierrez, Barry-Ryan et al. 2008a). Mejlholm and Dalgaard, 2002, speculated that essential oil antimicrobial activity could decrease in high fat food systems by dissolving in the lipid phases of the food, decreasing the lipophile activity on bacteria (Mejlholm and Dalgaard 2002). Essential oils were also shown to be more active at an acidic pH closer to 5 and decrease in activity when applied to products with a pH of 6 or 7 (Hsieh, Mau et al. 2001, Gutierrez, Barry-Ryan et al. 2008a). The bologna samples used in the experiment contained a high percentage of fat (25.71%), were composed of animal proteins, and had a pH of 6.29. This could explain why LM flourished in the inoculated, untreated samples as well as resisting antimicrobial activity of the essential oils. In addition, knowledge of LM growth and metabolism characteristics are limited when transferred to a MAP system. LM habitually transitions from an aerobic to anaerobic environment in nature and hosts, and could thusly display a spike of bactericidal resistance during the transition from an aerobic package or food product to the high or low oxygen environment of MAP systems (Lungu, Ricke et al. 2009).

Another rationalization for lackluster results is the dispersion ability of essential oils within the MAP system. Carbon dioxide is heavier than oxygen, utilized at 20% and 80% respectively within the packaging system. The likely presence of a CO_2 gaseous layer between the bologna samples and the oxygen atmosphere could partially disrupt an essential oil's ability to disperse toward the inoculated samples. Essential oils in Chapter 3 were tested with a microatmosphere method, simply requiring oils to disperse downward toward an inoculated agar. Essential oils in this trial were placed adjacent to the samples, requiring dispersion to drift upward and over the bologna slices. At least some components of essential oils were able to complete this dispersion due to the observation of discoloration (green or brown) on the bologna surface of most samples. However, the unidentified components needed for inhibition could have encountered decreased mobility, contributing to contrasting inhibition activity between *in vitro* (Chapter 3) and *in situ* (Chapter 4) studies.

4.5. Conclusion

When applied indirectly, as a volatile or gas dispersion, to an agar or food product essential oils are generally not guaranteed to inhibit bacteria in the same way a direct liquid application would, as seen by studies referenced in **Tables 2.1-2.3**. This experiment utilized 16 essential oils and essential oil constituents to evaluate inhibitory activity against *Listeria monocytogenes* on bologna samples in a MAP system. No oil or oil constituent achieved a mean practical reduction in *LM*, as seen by one $\log_{10}$ CFU/ml or 10% Change, aside from Eugenol (1.35ml) which could not be replicated. These

results are speculated to be a combination of sample formulation (fat, protein, pH), bacterial adjustment to MAP systems, gaseous dispersion, and activity transitions from a small scale *in vitro* study (Chapter 3) to a larger system sample.

Table 4.1: Essential Oil Volumes Converted from Petri Dish doses to MAP Tray Doses

| Conversion Equation	
$\dfrac{\text{MAP tray volume} \times \text{Microatmosphere Dose}}{\text{Petri dish volume}} = \text{MAP Dose}$	
Microatmosphere Dose, µl	MAP Tray Dose, ml
200	2.70
150	2.02
100	1.35
50	0.67
25	0.34
10	0.13

MAP: Modified Atmosphere Packaging, MAP tray volume: 1045.5ml, Petri dish volume: 77.5ml

Table 4.2: Mean % Change in inhibition activity of essential oils against *Listeria monocytogenes*

| Essential Oil	Volume			Standard Error	Students T-Test P value
	0.13mL	1.35mL	2.70mL		
Basil		5.650	-1.820	7.7	0.510
Bay		0.188	0.502	0.31	0.520
Bergamot	-0.565	-0.565		0.13	1.00
Chamomile		0.134	-2.076	2.4	0.528
Cinnamon		1.624	0.910	0.26	0.205
Citral	0.650	1.040		0.97	0.760
Clove Bud	16.309	0.910		14.94	0.490
Cumin Seed	0.640	0.320		1.1	0.819
Eucalyptus	1.216	-7.234		1.6	0.115
Eugenol		-10.181	-0.201	9.0	0.469
Geranium		-0.128	-0.272	0.6	0.937
Marjoram	-0.520	-3.899		0.71	0.113
Neroli Extract	9.219	0.128		8.2	0.467
Peppermint	-0.536	-5.157		0.5	0.048
Rosemary	-0.251	-1.946		0.44	0.161
Sage	-2.813	-2.009		1.4	0.677

T-test of % Change (+ is growth and – is reduction). % Change is the percentage of reduction or growth of *Listeria monocytogenes* colony counts on treated samples in comparison to untreated, inoculated control samples. 10% Change is equated to 1 log reduction/growth.

Figure 4.1: *Listeria monocytogenes* colony counts resulting after indirect exposure to Basil oil on MAP bologna slices stored at 24°C for 24h
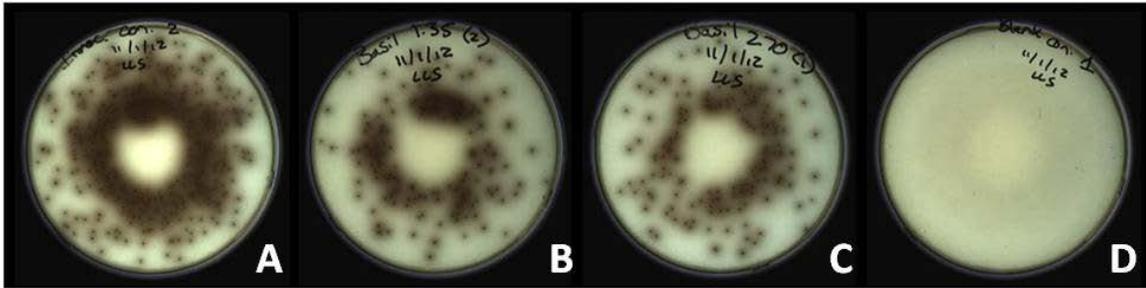
Previously inoculated bologna samples exposed adjacent to two volumes of Basil oil were collected, diluted, and plated on selective MOX agar for *Listeria monocytogenes* colony counts. Plated samples and colony counts are (A) inoculated control, 7.96 Log10 CFU/ml, (B) 1.35mL, 7.80 Log10 CFU/ml, (C) 2.70mL, 7.82mL, and (D) un-inoculated control, 0 Log10 CFU/ml.

Figure 4.2: *Listeria monocytogenes* colony counts resulting after indirect exposure to Bay oil volumes on MAP bologna slices stored at 24°C for 24h
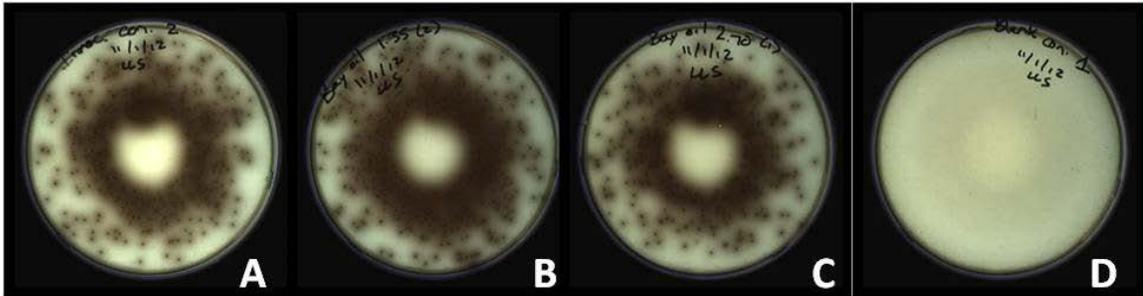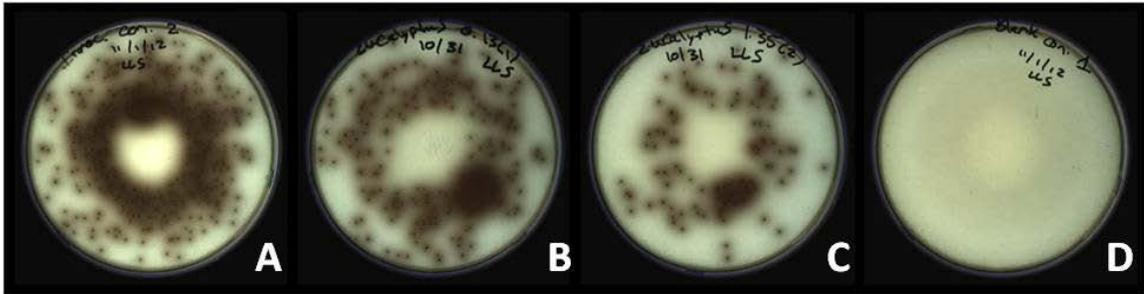

  Previously inoculated bologna samples exposed adjacent to two volumes of Bay oil were collected, diluted, and plated on selective MOX agar for *Listeria monocytogenes* colony counts. Plated samples and colony counts are (A) inoculated control, 7.96 Log10 CFU/ml, (B) 1.35mL, 8.0 Log10 CFU/ml, (C) 2.70mL, 7.98mL, and (D) un-inoculated control, 0 Log10 CFU/ml.

Figure 4.3: *Listeria monocytogenes* colony counts resulting after indirect exposure to Eucalyptus oil volumes on MAP bologna slices stored at 24°C for 24h

Previously inoculated bologna samples exposed adjacent to two volumes of Eucalyptus oil were collected, diluted, and plated on selective MOX agar for *Listeria monocytogenes* colony counts. Plated samples and colony counts are (A) inoculated control, 7.96 Log10 CFU/ml, (B) 0.13mL, 7.89 Log10 CFU/ml, (C) 1.35mL, 7.12mL, and (D) un-inoculated control, 0 Log10 CFU/ml.

Figure 4.4: *Listeria monocytogenes* colony counts resulting after indirect exposure to Rosemary oil volumes on MAP bologna slices stored at 24°C for 24h
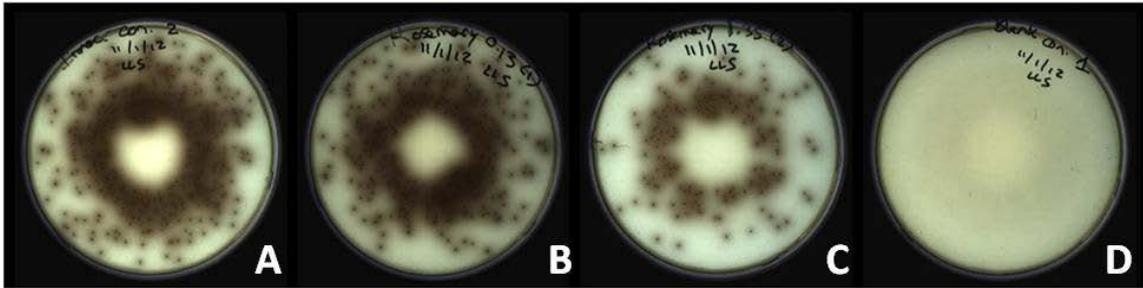

Previously inoculated bologna samples exposed adjacent to two volumes of Rosemary oil were collected, diluted, and plated on selective MOX agar for *Listeria monocytogenes* colony counts. Plated samples and colony counts are (A) inoculated control, 7.96 Log10 CFU/ml, (B) 0.13mL, 7.98 Log10 CFU/ml, (C) 1.35mL, 7.81mL, and (D) un-inoculated control, 0 Log10 CFU/ml.

CHAPTER 5

OVERALL CONCLUSIONS

Overall, various levels of inhibition activity were observed by 16 essential oils when applied to *LM* using the microatmosphere method at three storage temperatures (37°C for 24h, 24°C for 24h, 4°C for 10 days), shown in **Tables 3.2-3.4**. Samples stored at 24°C for 24h generally displayed enhanced inhibition activity, with the exception of Chamomile and Geranium oil, seen in **Table 3.3**. However, very little inhibition activity was detected when the oils were exposed to *LM* inoculated bologna slices packed in MA (80% O_2, 20% CO_2) and stored at 24°C for 24h. None of the selected oils, disregarding Eugenol, achieved a mean one $\log_{10}$ CFU/ml reduction in *LM*. Eugenol (1.35ml) did achieve a mean one $\log_{10}$ CFU/ml reduction, but it could not be replicated. Peppermint oil ($P < 0.048$) displayed significant differences between application volumes (0.13ml, 1.35ml) though no practical $\log_{10}$ CFU/ml reduction was observed. Generally, many oils have displayed varied levels of antimicrobial activity *in vitro* and *in situ*, shown in **Tables 2.1-2.3**. Decreased antimicrobial activity by essential oils is known to occur when applied to food products, and could be attributed to many factors (Burt 2007). Protein, fat, and pH characteristics of a food product may change the efficacy of essential oils. Slightly acidic products (pH 5) have been shown to support greater antimicrobial activity of essential oils, as compared to a more neutral pH of 6 or 7. In addition, increased animal and plant protein could enhance the growth of LM, while increased animal and plant fat percentages could interfere with an essential oils' ability to act as a lipophile to

bacteria (Mejlholm and Dalgaard 2002, Gutierrez, Barry-Ryan et al. 2009). The bologna utilized in Chapter 4 contained a high percentage of animal protein and fat (25.71%) with a less acidic pH (6.29), and could be a major reason for decreased antilisterial activity. Also, essential oil dispersion in Chapter 3 required only downward motion, while dispersion in Chapter 4 required movement upward and toward the samples. The possible presence of a CO_2 layer with the MAP system could also have prevented some inhibition activity. This research suggests that the selected essential oils, while effective *in vitro*, may not be ideal in RTE meat food models similar to bologna. In future research, essential oils employed in Chapter 3 could be directed toward foods with lower percentages of fat and protein, such as fruits and vegetables. The packaging system must also offer minimal interference with the indirect mechanism of action by essential oils or enhance antimicrobial activity, one possibility being incorporation of the oil vapors directly into gas mixtures.

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