Evaluating the Antimicrobial Efficacy of White Mustard Essential Oil against

Salmonella

by

John Adam Porter

A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 5, 2017

Keywords: Food Safety, Salmonella, Natural Antimicrobials, White Mustard Essential
Oil, Food Application

Copyright 2017 by John Adam Porter

Approved by

Emefa Monu, Assistant Professor of Food Science
Amit Morey, Assistant Professor of Food Science
Tung-shi Huang, Professor of Food Science

Abstract

Currently, it is estimated that non-typhoidal *Salmonella* causes approximately 1.2 million

illnesses and 450 deaths per year in the United States. Reduction of *Salmonella*-related

foodborne infections can be achieved through reducing or eliminating the pathogen by

antimicrobials. Essential oils in combination with other antimicrobials can be added to

raw poultry products to reduce the levels of the organism below the infectious dose for

healthy individuals. The purpose of this study was to investigate the synergy between

WMEO and carvacrol or thymol and its potential to be used in the control of *Salmonella*.

Additionally, the activity of WMEO and carvacrol were tested against *Salmonella* in

ground chicken to see the effect a food product would have on the efficacy of the

antimicrobial activity. The results showed that an additive effect was achieved between

WMEO and carvacrol as well as thymol. In addition, 0.75% WMEO and 0.1% carvacrol

combination had a bacteriostatic effect against *Salmonella* at a temperature abuse of 10

°C for 7 days. At 4 °C, the combination of 0.75% WMEO + 0.1% carvacrol had a 0.6 log

CFU/g less count of *Salmonell*a when compared to the positive control. The application

of the antimicrobials controlled the growth of *Salmonella* by delaying the exponential

phase at temperature abuse and reducing levels of *Salmonella* to be less than the positive

control at 4°C. The use of these antimicrobials show potential in reducing levels of

Salmonella below infectious doses and controlling the growth of the organism under

temperature abuse conditions in raw poultry products. Further research is needed to

investigate the toxicity of the compounds as well as the most efficient way to apply it to a

food product to maximize antimicrobial activity.

Acknowledgements

I first want to thank my major advisor Dr. Emefa Monu, for her guidance and support through my graduate studies at Auburn University. Her patience and knowledge of food microbiology has made the completion of this master's project possible. Additionally, I would like to thank my graduate study committee members of Food Science, Dr. Tung-shi Huang and Dr. Amit Morey for their time and expertise in food microbiology and processing.

I would like to express my gratitude to my laboratory co-workers Michelle Hayden, Avery Smith, Alejandra Abrego, Mingyu Qiao, Tian Ren, and Shelli Laskowitz for their assistance throughout my research. Also want to thank my brother Allen Porter for constantly pushing me and believing that I could do anything. Finally, I would like to thank my parents Gary and Rhonda Porter, and my girlfriend Madeline Dean for their support and motivation throughout my life and academic career.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

AITC	Allyl Isothiocyanate
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BHA	Butylated Hydroxyanisole
BHT	Butylated Hydroxytoluene
BPW	Buffered Peptone Water
CD	Cyclodextrin
CFAB	Citrus Flavanoid and Acid Blend
CFU	Colony Forming Unit
CIDT	Culture Independent diagnostic test
DMSO	Dimethyl Sulfoxide
E.coli	*Escherichia coli*
EO	Essential Oil
FDA	Federal Drug Administration
FICI	Fractional Inhibitory Concentration Index
GAP	Good Agricultural Practices
GRAS	Generally Recognized as Safe
4-HBITC	p-Hydroxybenzyl Isothiocyanate
HD	Hydrodistillation
HPP	High Pressure Processing
ITC	Isothiocyanates

kGY	Kilograys
LAE	Lauric Arginate
MAHD	Microwave-assisted Hydrodistillation
MAP	Modified Atmospheric Packaging
MIC	Minimum Inhibitory Concentration
MHB	Mueller-Hinton Broth
Nalidixic Acid	NAL
OD	Optical Density
PAA	Peracetic Acid
PW	Peptone Water
PPM	Parts Per Million
SDE	Simultaneous Distillation
S. Enteritidis	*Salmonella* Enteritidis
SFE	Supercritical Fluid Extraction
S. Hartford	*Salmonella* Hartford
S. Heidelberg	*Salmonella* Heidelberg
S. Kentucky	*Salmonella* Kentucky
S. Montevideo	*Salmonella* Montevideo
S. Newport	*Salmonella* Newport
SNK	Student-Newman Keuls
S. Orion	*Salmonella* Orion
SPP	Species

S. Senftenberg	*Salmonella* Senftenberg
S. Typhimurium	*Salmonella* Typhimurium
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TT	Tetrathionate Broth
USDA	United States Department of Agriculture
WMEO	White Mustard Essential Oil
XLT4	Xylose-Lysine-Tergitol 4
ZOI	Zones of Inhibition

Chapter 1: Introduction

1.1 Background

Currently, it is estimated that non-typhoidal *Salmonella* causes approximately 1.2 million illnesses and 450 deaths per year in the United States (CDC 2015). In addition, *Salmonella* remains as the leading cause of foodborne illness from the consumption of raw and undercooked chicken products. Current control methods of *Salmonella* in raw chicken products include the use of peracetic acid (PAA) in post-slaughter process within the plant, modified atmospheric packaging (MAP) and temperature. Recently, consumers have shown concern about artificial and synthetic ingredients in the food industry, driving the current trend of naturally derived ingredients and antimicrobials (Burt 2004). Consumer trends have presented more of a challenge to the food industry to reduce the occurrence of foodborne illness while keeping products free of synthetic preservatives. Therefore, the food industry is looking for more natural preservatives to meet the consumers' standards and desire for more natural food products.

Essential oils (EO) are natural compounds derived from plants that have been studied for years for their different chemical and biological properties. Of these properties, some essential oils possess antimicrobial activity. White mustard essential oil (WMEO), derived from the plant*Sinapis alba* L., is an essential oil that has shown *in-vitro* antimicrobial activity against *Salmonella* Enteritidis, *Escherichia coli*, *Listeria monocytogenes*, *Staphylococcus aureus*, *Clostridium perfringens*, *Pseudomonas aeruginosa* and *Campylobacter jejuni* (Techathuvanan et al. 2014). WMEO shows

potential as a natural preservative to be used in variety of products due to its broad antimicrobial activity.

In addition to, WMEO and other EO's possess strong sensory properties and instability at warm temperatures, and when used in food products they require higher concentrations than what is used in vitro to inhibit food pathogens. This has been a major issue for the broad use of EO in foodstuffs. EO's have been used in combination with other antimicrobials to reach a synergistic effect and has been proven to be effective in reducing the concentrations needed to inhibit foodborne pathogens. WMEO has been shown to be more stable at refrigeration and frozen temperatures making its application to a refrigerated or frozen product ideal (Ekanayake et al. 2006). The main antimicrobial component, 4-HBITC (p-hydroxybenzyl isothiocyanate) has shown stability in 700W and 1,100 W microwave cooking conditions. Not only was there a reduction of *Salmonella* spp., at the end of the cook time the presence of 4-HBITC was not detected (David et al. 2012). This further implicates the potential of this compound to be used in controlling *Salmonella* spp. in a raw or microwavable product without changing the sensory profile.

1.2: Purpose of Study

The purpose of this study is to investigate the antimicrobial activity of WMEO against *Salmonella* spp. and assess its efficacy in a raw meat product. To achieve this goal, several objectives are listed: 1) to investigate sensitivities to WMEO between *Salmonella* enterica serovars; 2) to determine the minimum inhibitory concentrations of WMEO, carvacrol and thymol against *Salmonella*; 3) to assess the binary combinations of WMEO, carvacrol and thymol against *Salmonella* as antagonistic, additive or

synergistic; 4) to assess the efficacy of WMEO and carvacrol in controlling *Salmonella* in ground chicken at 4 °C and 10 °C.

1.3 Significance of Study

The safety of food products is a major concern in the food industry. Consumers desire less artificial preservatives and more natural food products. The food industry requires more natural antimicrobials as replacements for commonly used artificial preservatives for use in clean label products and to meet consumer needs, while still maintaining the safety of the food. Essential oils remain a popular avenue of research in controlling foodborne pathogens due to many possessing antibacterial and antifungal properties. WMEO has shown potential in controlling *Salmonella* in food products. More specifically, in the poultry industry where *Salmonella* still remains a problem, it could decrease the number of outbreaks per year regarding raw and processed chicken products. Evaluating the antimicrobial activity of this compound in ground chicken could unveil a possible preventive control that can be used to keep products safe from post-process contamination and in temperature abused conditions.

Chapter 2: Literature Review

2.1: *Salmonella* and Outbreaks

      Salmonella is a facultative anaerobe and a gram-negative, rod-shaped bacterium that causes salmonellosis (Madigan et al. 2012). Salmonellosis is an infection that causes gastroenteritis 8 to 72 hours after exposure/infection and common associated symptoms include headache, fever, abdominal cramps, diarrhea and vomiting (Madigan et al. 2012, Montville et al. 2005). In most cases, individuals can recover from *Salmonella* infections without treatment in 2-5 days (Madigan et al. 2012). However, some cases are severe usually in elderly, children and immunocompromised individuals, and require medical treatment as they are more susceptible to *Salmonella* than healthy individuals (Montville et al. 2005). The severity of the infection is dependent on the amount of organism ingested and the age and current health of the individual. According to FoodNet, *Salmonella* has been the number one bacterial pathogen that causes the most foodborne illness in the United States since the Foodborne Disease Active Surveillance Network (FoodNet) was established in 1996 (CDC 2017[a]). However, a recent study in 2016 by the CDC using culture independent diagnostic tests (CIDT) has revealed that *Campylobacter* is now estimated to cause more illnesses than *Salmonella* (CDC 2017[b]),although, *Salmonella* remains in the top 10 foodborne pathogens causing the 2nd most cases by a bacterium. Currently, it is estimated that non-typhoidal *Salmonella* causes approximately 1.2 million illnesses and 450 deaths per year in the United States (CDC 2015[a]). In 1997 there were an estimated 1.4 million illnesses per year and 300 deaths in the United States from non-typhoidal *Salmonella* (Mead et al. 1999). Although, *Salmonella* infections have

stayed consistent over the years, the organism still remains a significant health problem. In the past 10 years, several foodborne outbreaks have occurred as a result of *Salmonella* contamination. *Salmonella* has been associated with a variety of foods including vegetables, beef, turkey, chicken, pork, fruit and nuts. During the years 1998-2008, the four most commonly isolated serotypes of *Salmonella enterica* were Enteritidis, Typhimurium, Newport and Heidelberg. These serotypes were responsible for 66% of salmonellosis outbreaks that occurred in this 10 year period (Jackson 2013). The outbreaks discussed in this paper will be within the last five years and focus on the six strains of *Salmonella enterica* relevant to this study. These strains include *S.* Enteriditis, Typhimurium, Heidelberg, Orion, Senftenberg, Hartford, Kentucky and Montevideo. *S.* Enteriditis, Typhimurium, and Heidelberg outbreaks have been primarily associated with meat products. In 2015, Barber foods had to recall their chicken products due to contamination of *S.* Enteritidis. The chicken products included raw frozen stuffed chicken entrees. Nine people became ill, four of which had been infected with an antibiotic resistant strain of *S.* Enteritidis from the poultry products. The antibiotic strains isolated were resistant to ampicillin and tetracycline (CDC 2015[b]). In 2013, there was an outbreak with Foster Farms chicken products contaminated with *S.* Heidelberg; 634 cases were reported and 200 hospitalized. Isolates were collected and some of the infected patients had drug resistant strains of *S.* Heidelberg (CDC 2014). Another outbreak caused by *S.* Typhimurium associated with ground beef occurred in 2013. Six states were involved resulting in 22 illnesses and 7 hospitalizations (CDC 2013). In addition, *S.* Enteriditis was the cause of another outbreak associated with ground beef in 2012. The outbreak had 46

cases across multiple states and 12 people were hospitalized (CDC 2012[a]). In 2011, *S*.

Heidelberg was associated with ground turkey and infected 136 people across 34 states.

Of the 136, 37 were hospitalized and 1 death resulted from the outbreak. These recent

outbreaks show that *Salmonella* remains a problem in poultry products as well as other

meat products. *Salmonella* is zoonotic and ubiquitous which is why it is commonly

recovered from animal products. *Salmonella* is not naturally found on fruit or produce but

can contaminate these products due to improper Good Agricultural Practices (GAP's).

Cross-contamination from animals and the environment can place *Salmonella* onto raw

produce and fruit. In 2012, cantaloupe was recalled from a producer in Indiana for

contamination with *S*. Typhimurium and Newport. The outbreak resulted in 294 cases, 94

hospitalizations and 3 deaths; *S*. Typhimurium was responsible for 228 of the cases.

Environmental sampling showed the packing facility was contaminated with *S*.

Typhimurium and *S*. Newport at several locations allowing transmission of the organism

onto the produce (CDC 2012[b]). These recent outbreaks highlight continued prevalence of

Salmonella throughout the food chain, and the need for interventions and antimicrobials

that can decrease the transmission of these bacteria.

2.2: Essential Oil

Natural compounds known as essential oils show potential in controlling

Salmonella and other foodborne pathogens in food products. EO's are volatile and

hydrophobic secondary metabolites derived from plants and some of these compounds

serve to protect the plant from outside invaders such as microorganisms. In addition,

EO's have been known to possess flavor, fragrance, antimicrobial and antioxidant

properties and are primarily utilized for their flavor characteristics in the food and beverage industry (Burt 2004). However, in the last 30 years, much focus has shifted to the antimicrobial activity of some EO's. One reason is concern about harmful side effects from artificial preservatives traditionally utilized, creating an immediate need for the development and discovery of effective natural preservatives. EO's fit that role since it is natural and most have strong antimicrobial properties. Some advantages of using natural preservatives to control foodborne pathogens is that they are more consumer friendly than artificial preservatives, increase the shelf-life of natural products and fits the current trend of green consumerism (Burt 2004). Several EO's from different plant sources have been tested against common foodborne pathogens to see the extent of their antimicrobial activity. Oregano, thyme, cinnamon, and clove are the most commonly studied EO's and have shown strong antimicrobial activity (Lambert et al. 2001, Friedman et al. 2004, Solomakos et al. 2008). These contain phenolic antimicrobial compounds that have been proven powerful antimicrobials as their major constituents, which is why they are studied. Carvacrol and thymol are phenolics found in oregano and thyme oil. Cinnamon's main antimicrobial component is trans-cinnamaldehyde and clove contains eugenol. All of these constituents have been studied to great extent (Burt 2004, Consentino et al. 1999, Dorman et al. 2000).

Currently, the food industry is looking for alternatives to artificial preservatives. Artificial preservatives that have been reported to potentially cause harmful effects include: nitrates, benzoates, sulfites, sorbates, parabens, formaldehyde, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Anand et al. 2013). All of

these compounds have restrictions on the amount that can be used in a food for preservation. Recently, sodium benzoate was found to have clastogenic, mutagenic and cytotoxic effects in human peripheral blood lymphocytes. This research has caused concern about the possible toxicity of sodium benzoate and other benzoate preservatives which are commonly used in seafood, pickles, fruit juices, soft drinks, sauces and edible coatings (Carocho et al. 2014). If further research concludes that these compounds are unsafe to use, alternative antimicrobials will need to be ready to take their place in order to prevent future outbreaks. Sorbates are primarily used to control mold and yeast to prevent spoilage in baked goods, cheese, wine and yogurt. However, sodium sorbate may have toxic effects against human blood cells and potential to be oxidized into a mutagenic compound called 4,5-oxohexonoate (Carocho et al. 2014). A natural preservative with no toxic effects might be needed to replace sorbates in the future for safety reasons. In addition, using natural preservatives will fit the current need of more natural ingredients demanded by consumers. Nitrates are another synthetic antimicrobial that has been found to potentially cause hazardous effects. It is used in processed meats and fermented meats to prevent growth of *Clostridium botulinum*. The problems with nitrates are it can be converted to nitrites by the stomach which have been shown to potentially be altered into carcinogenic nitrosamines. Another artificial preservative is sulfites, which are used to reduce microbial loads in food and prevent spoilage. It is also used to prevent oxidation in foods to preserve the color and prevent off-flavors. However, sulfites can cause allergic reactions in some individuals. Data through 1985, reported 250 cases related to

allergic reactions to sulfites in the United States. Sulfite use as a preservative is currently limited in food due to many allergic reactions (Anand et al. 2013).

2.3: Types of Natural Plant Antimicrobials Compounds

Secondary metabolites that are extracted from plants can be divided into different classes. Terpenoids, phenylpropenes and isothiocyanates are some classes of plant metabolites that have exhibited antimicrobial activity against a broad spectrum of microorganisms, including *Salmonella*.

Terpenoids

Terpenoids are terpenes that have had their structure modified usually through the addition of oxygen molecules via enzymes. However, terpenes can become terpenoids through re-location of methyl groups or removal of methyl groups and re-arrangements into cyclic structures. The type of terpenoid synthesized depends on the precursor terpene and the biochemical modification. Terpenes are organic compounds made of isoprene units (C_5H_8) which can vary in length. Types of terpenes are monoterpenes ($C_{10}H_{16}$), sesquiterpenes ($C_{15}H_{24}$), diterpenes ($C_{20}H_{32}$), triterpenes ($C_{30}H_{40}$) ect. (Hyldegaard et al. 2012). Carvacrol and thymol are plant secondary metabolites that are categorized as phenolic monoterpenoids. Thymol inhibits growth of microorganisms by interacting with the cell membranes (Hyldegaard et al. 2012). The exact mechanism is still not fully understood. However, studies reveal that thymol causes increased membrane permeability resulting in leakage in cellular contents and loss of membrane potential (Lambert 2001, Helander 1998). Carvacrol works in a similar manner affecting the cytoplasmic membrane. The interaction is thought to increase the fluidity and

9

permeability of the cytoplasmic membrane. This puts stress on the cells due to the loss of H^+, K^+ and adenosine triphosphate (ATP) which are needed for growth in bacterial cells (Hyldegaard et al. 2012). It has been established that the minimum inhibitory concentration (MIC) of carvacrol and thymol against *Salmonella* typhimurium is 1mmol (Helander et al. 1998; Nazer et al. 2005; Olasupo et al. 2003). These compounds are possible preservatives to be used to control *Salmonella* in food.

Phenylpropenes

Phenylpropenes are another class of essential oil compounds that are derived from the amino acid phenylalanine in plants. EO components that fall under this class that have been extensively studied are cinnamaldehyde and eugenol.

Cinnamaldehyde is the main antimicrobial component in cinnamon. As mentioned previously it is a phenolic compound with an aldehyde side chain. Several mechanisms of this compound have been proposed and hypothesized but there is still much that is unknown of how it works to inhibit bacteria. It has been suggested that the aldehyde component of cinnamaldehyde might impede DNA and protein function. Another mechanism of how cinnamaldehyde can inhibit the growth of bacteria is increasing the permeability of the cytoplasmic membrane. The MIC of cinnamaldehyde is 3 mmol for both *E. coli* and *S*. Typhimurium. However, compared to natural antimicrobials carvacrol and thymol are more potent to *S*. Typhimurium (Helander et al. 1998).

Eugenol is the main compound that is found in clove essential oil. It is a phenolic compound that is similar to guaiacol except with an allyl side chain. Eugenol has shown antimicrobial activity against *S*. Typhimurium, *E. coli* and *L. monocytogenes*. The

mechanism of action is similar to carvacrol as it disrupts the cytoplasmic membrane

causing a change in permeability (Gill et al. 2006). One study established the MIC of

eugenol to be 3mmol and 2.5mmol for *S*. Typhimurium and *E. coli* (Olasupo et al. 2003).

Although it shows potential in being used as an antimicrobial carvacrol and thymol have

been shown to be more effective at inhibiting *S*. Typhimurium and *E. coli*.

Isothiocyanates

Isothiocyanates are essential oil compounds derived from mustard, broccoli,

cabbage, horseradish and turnips which, belong to the mustard family (*Brassicaceae*).

Glucosinolates are compounds that yield isothiocyanates through a hydrolytic reaction

via myrosinase. Four possible products can be formed from this reaction: isothiocyanates,

thiocyanates, nitriles and cyclic compounds. Injury of plant tissues triggers this reaction

and forms isothiocyanates (ITC) as a defense mechanism. According to literature, the

absence of myrosinase associated proteins guarantees formation of ITC's (Angelino

2015). The type of ITC that is formed is dependent on the R-side chains on the parent

glucosinolate. For example, horseradish and brown mustard plants contain allyl

glucosinolate and both produce allyl isothiocyanates. In addition, other isothiocyanates

with different R-groups have been sourced from horseradish and brown mustard plants.

White mustard oil plants produces only p-hydroxy-benzyl methyl isothiocyanate (4-

HBITC) shown in figure 2.3.1. The chemical structures of glucosinolates vary between

plants within the *Brassicaceae* family and the mode of action for isothiocyanates is not

definitively known (Delaquis 1995). Allyl isothiocyanate shown in figure 2.3.1 is thought

to interact with the cell membranes of bacteria causing leakage of ions and ATP

(Hydellgard 2012; Lin 2000). Allyl isothiocyanate has been the most studied mustard oil in the class of isothiocyanates and why there is more information available compared to other isothiocyanates.

Figure 2.3.1 p-hydroxybenzylisothiocyanate (A) (4-HBITC) and allyl isothiocyanate (B) (National Center for Biotechnology Information)

(A)   (B)
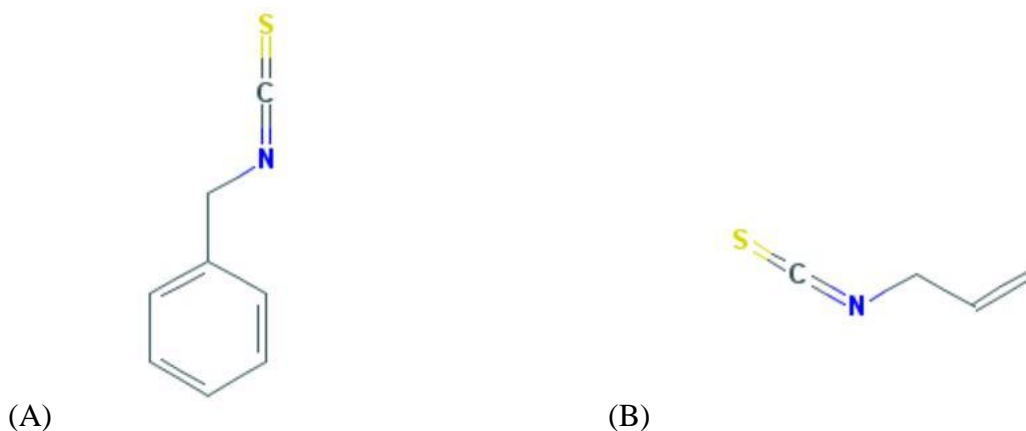
White mustard oil is another essential oil that contains a compound in the isothiocyanate family. It is derived from the white mustard seed (*Sinapis alba* L.). The main antimicrobial component in white mustard oil is 4-HBITC and is formed through a hydrolytic reaction between the glucosinolate sinalbin and thioglucoside glucohydrolase, a myrosinase enzyme (Ekanayake et al. 2006). It shows potential as a natural preservative and has been shown to be inhibitory to both gram positive and gram-negative bacteria.

White Mustard Essential Oil

One study demonstrated that WMEO was inhibitory against *Salmonella* Enteritidis at concentrations of 500 and 1000ppm 4-HBITC up to 24h and 50h, respectively (Monu 2014). Currently, the minimum inhibitory concentrations (MIC) for WMEO has only been determined for *Escherichia coli*, *S*. Enteritidis, *Enterobacter aerogenes*, *Bacillus*

cereus, *Staphylococcus aureus* and *Listeria monocytogenes* at the following

concentrations of 0.15%, 0.15%, 0.15-0.175%, 0.025%, 0.05% and 0.1% respectively

(Techathuvanan et al. 2014). Of the foodborne pathogens tested *Bacillus cereus* is the

most susceptible to WMEO followed by *Staphylococcus aureus*, *Listeria monocytogenes*,

S. Enteritidis, *E.coli* and *Enterobacter aerogenes*. In addition to in vitro studies, WMEO

has also shown success in application to a food product. One study applied oil

concentrations containing 250 to 750ppm 4-HBITC to a frozen sauce inoculated with

Salmonella spp. The application resulted in a reduction of up to 2.7 log CFU/g, but high

concentrations of WMEO were needed to achieve an inhibitory effect against *Salmonella*

(David et al. 2013). However, after heating, the presence of the main sensory component

4-HBITC in mustard oil was not detected in the sauce according to David et al. 2013. The

volatility of the mustard oil shows potential to be used in reducing risk of illness in

microwaveable products without compromising the flavor profile.

2.4: Composition of Essential Oils

The composition of these oils can be complex and can contain more than sixty

individual components. Oregano and thyme essential oils have similar compositions.

Both contain thymol and carvacrol as their major constituents but oregano has higher

concentrations of carvacrol and thyme contains higher concentrations of thymol.

Environmental conditions can impact the levels of thymol and carvacrol. The

concentrations of these components vary depending on the time of harvest and it has been

shown that thyme yields higher concentrations of thymol and carvacrol during flowering

or immediately after (Burt 2004; McGimpsey 1994; Russo 1998; Senatore 1996). It has

been shown that spacing of plants also has an effect on oil yields. According to Badi 2004, spacing thyme 15cm apart showed optimal yields of thymol. Additionally, extraction methods can also effect the efficacy of the EO. The conventional extraction methods for essential oils are steam distillation, hydrodistillation (HD) and simultaneous distillation (SDE) methods. However, newer methods are arising in an attempt to increase yield, reduce costs and extraction time. Supercritical fluid extraction (SFE) shows promise in obtaining phenolic compounds such as thymol and carvacrol utilizing carbon dioxide. It has been shown that SFE produces higher amounts of thymol and carvacrol with better precision than SDE. In Diaz-Maroto et al. 2005, SFE extraction produced an oil composition from *Thymus vulgaris* L. of approximately 61%, 9% and 8.5% of thymol, carvacrol and p-cymene, respectively. In comparison to SDE extraction only produced 31%, 5% and 33% thymol, carvacrol and p-cymene respectively. In conclusion, it was found that SFE is more efficient at extracting oxygenated terpenes and high boiling point components such as thymol and carvacrol than SDE. In contrast, SDE is more efficient at extracting terpene hydrocarbons (Diaz-Maroto et al. 2002, 2005). SFE's major setback is cost even though it has shown higher efficiency when compared to other processes such as SDE. Ultimately, the choice of the extraction process will be determined by the use of the essential oil and the cost. Solvent-free microwave extraction (SFME) is another method that has been shown to produce higher yields of thymol and carvacrol from *Origanum vulgare* L. than HD (Bayramoglu et al. 2008). Furthermore, the extraction time was significantly reduced as well as the cost of extraction using SFME compared to HD. These results are in agreement with another study that compared microwave-assisted

hydrodistillation (MAHD) to the conventional HD method in analyzing *Thymus vulgaris L* (Golmakani et al. 2008). For WMEO, supercritical fluid extraction with CO_2 has been used to extract from the white mustard seed but is a costly process. However, cold press method utilizing ethyl acetate as the dissolving agent has been shown to be effective at extracting WMEO from the white mustard seed containing concentrations of up to 26% 4-HBITC (Ekanayake et al. 2006). The cold press method costs less than the supercritical extraction utilizing CO_2 and could help to make the commercial production of WMEO as an antimicrobial viable. In commercial production, composition yield, efficiency, cost, extraction time and origin of plant will be important factors to consider for providing effective and consistent compositions of essential oils to be used in food preservation.

2.5: Application of Essential Oils in Food Products

Most essential oils have generally recognized as safe (GRAS) status in foods and of the essential oils previously mentioned, cinnamaldehyde, oregano and thyme essential oil have GRAS status (FDA 2017). The safety and toxicity of white mustard, carvacrol and thymol are to be determined. Application of essential oils to food products is still in the developing stages and of the application methods edible films is the most prominent delivery system used to apply essential oils onto a food product. However, this method of delivery will only impact surface pathogens on the products thus limiting the application to certain foods. Examples of these food types mostly include raw meat or produce, the types of foods in which in general, *Salmonella* tends to be a problem. Use of edible films with essential oils could help to reduce the number of *Salmonella* outbreaks associated with these types of food. These films can be made from polysaccharides, protein and

lipids. Some examples of the components that have been used in films are soy, whey, casein and milk protein, chitosan, pectin, gums, wheat gluten and cellulose derivatives (Falguera et al. 2011; Seydim et al. 2006). Some films seem to work better in combination with essential oils than others. One study used whey protein films and oregano essential oil to test the viability to inhibit several foodborne pathogens. At a concentration of 1% the oregano essential oil had no effect on all test organisms. However, at 2% it showed zones of inhibition (ZOI) at 33mm for *S. aureus* and 19mm for *E. coli* O157:H7 (Seydim et al. 2006). Stronger inhibitory effects have been seen in combining oregano essential oil with soy protein edible films. It was shown that soy protein films incorporated with 1% oregano essential oil had a ZOI of 27.5mm against *S. aureus* and 35mm against *E. coli* O157:H7 (Emiroglu et al. 2010). It is obvious that the composition of the film will play a factor in the efficacy of the essential oil against foodborne pathogens in a given product. Another film base that shows promise is a polysaccharide known as chitosan. Chitosan is derived from chitin which is found in the exoskeleton of crustaceans and fungal cell walls (Falguera et al. 2011). Chitosan based film incorporated with 1% and 2% oregano essential oil reduced *L. monocytogenes* by 3.6 to 4 log CFU/sandwich. In addition, the chitosan-oregano film reduced *E. coli* by 3 log CFU/sandwich (Zivanovic et al. 2005). It should be noted that chitosan can exhibit activity on some level against microorganisms. In the study mentioned previously, it reduced *L. monocytogenes* by 2 log CFU/sandwich without the essential oil (Zivanovic et al. 2005). Further research should be done to determine the best film compositions for essential oils and various food products. As the effectiveness of the films could vary

depending on the essential oil being used and the target food product (Falguera et al. 2011).

Another application of essential oils in food are the use of encapsulation via cyclodextrins (CD). Cyclodextrins were first discovered and isolated by Villiers in 1891. CD's were the degradation product of *Bacillus amylobacter* breaking down starch molecules. However, it was Schardinger who characterized the CD's as cyclic oligosaccharides. Since its discovery, CD's have been used in the food and beverage industry as carriers for flavor compounds and other sensitive ingredients. Some of the benefits associated with using CD's as carriers are improved physical and chemical stability of the target compound and masking of odor (Cabral Marques et al. 2010). One of the main issues with essential oils is the strong sensory properties they possess and their tendency to diffuse into the lipid portions of the food. CD's have been proven to reduce the sensory effects in food and increase the antimicrobial action of EO compounds in vitro (Hill et al. 2013; Cabral Marques et al. 2010). Hill and others (2013), showed that >0.1% of eugenol and clove bud extract was needed to inhibit the growth of *S. typhimurium* at 35°C for 24h incubation. However, when eugenol and clove was complexed with beta-CD's, the MIC concentrations of *S.* Typhimurium at 35°C were 0.069% and 0.028%, respectively. The increase in antimicrobial activity could help reducing the sensory impact of the EO by lowering the amount needed to inhibit the target pathogen. In addition, these CD-complexes have been known to mask the sensory attributes of compounds which could increase the sensory threshold of EO application to a food product (Cabral Marques 2010). The type of cyclodextrins are also important as it

can influence the antimicrobial activity of a compound. For example, one study formed complexes with alpha, beta and hydroxyl-propyl-beta CD's using EO's. It was found for carvacrol that alpha and hydroxyl-propyl beta CD was more effective showing lower MIC's for *E. coli* and *Staphylococcus aureus* than beta CD (Liang et al. 2012). These recent developments in delivery systems such as cyclodextrins and edible films have potential in effectively incorporating more natural antimicrobials into food products.

2.6: Obstacles of EOs as Food Antimicrobials

Although many EO's exhibit antimicrobial activity and show potential, there are still obstacles preventing successful application to a food product. One obstacle concerns the hydrophobic nature of the antimicrobial compounds found in EO's. Foods that have high fat content greatly reduce the efficacy of EO's. The exact mechanism of how fatty foods decrease the antimicrobial activity is not definitive. However, it is thought that the essential oil diffuses into the lipid layer of the food away from the aqueous phase. (Donsi et al. 2011, Weiss et al. 2009) Pathogens can be found in both lipid and aqueous phases. This can reduce the efficacy of the antimicrobial by allowing it to only affect the population of bacteria in the lipid phase leaving bacteria in the aqueous phase unaffected. The hypothesized mechanism explains why in vivo applications of EO's require higher concentrations to inhibit organisms than what is found in vitro. On the contrary, experiments have shown that foods with low pH and high protein content improve the antimicrobial activity of EO's (Gutierrez et al. 2008). Another obstacle is the strong sensory property that EO's possess. It is ideal to add preservatives to a food product without compromising the organoleptic profile. Generally, EO concentrations needed to

inhibit bacteria in a food product will alter the flavor profile of the food (Busatta et al. 2008, Nielsen et al. 2000). Current strategies used to reduce the concentration needed for essential oils include combining antimicrobials, applying lower temperatures and lowering pH and oxygen levels. These options refer to a concept called Hurdle Technology. Hurdle Technology describes how growth of microorganisms can be suppressed by adding multiple hurdles or strategies the organism has to overcome in order to reach exponential growth (Burt 2004).

Lowering the pH of the food is known to increase the effectiveness of the essential oils. The hydrophobicity of the oil increases with a lower pH allowing easier transmission into the lipid portion of the bacterial cell membrane (Burt 2004; Juven et al.1994; Negi 2012). This can be seen in an experiment conducted with allyl isothiocyanate. Luciano and Holley 2009, tested allyl isothiocyanate against *E. coli* O157:H7 cocktail in Luria-Bertani broth with different pH concentrations. It was found that the minimum inhibitory concentration decreases with lower pH. Ally isothiocyanate has more stability at acidic pH's which contributes to its increased antimicrobial activity (Tsao et al. 2000).

Applying refrigeration temperatures to food products is an effective way to extend the lag phase of mesophilic foodborne pathogens, such as *Salmonella* spp. It will slow the growth and recovery rate of the organism. Lower temperature in combination with an antimicrobial may cause too much stress for the organism leading to increased inhibition or cell death. Case in point, one study combined refrigeration, oregano essential oil and modified atmospheric packaging to hinder the growth of spoilage organisms on chicken

breasts. Combining all three resulted in a shelf life extension of more than 20 days stored at 4 °C. It is a prime example of how hurdle technology can hinder the growth of microorganisms. Some studies have suggested that refrigeration temperatures has a protective effect on *Salmonella* spp. in food (Tassou et al.1995). In contrast, it has been found by Ekanayake and others that 4-HBITC, active component in white mustard oil, is more stable at lower temperatures in acidic solutions. The increased stability could contribute to higher antimicrobial activity against foodborne pathogens under acidic and refrigerated conditions. Also oregano and thymol can maintain optimal antimicrobial activity over a broad temperature range (Burt and Reinders 2003). Therefore, these components could be used in combination with white mustard essential oil to ensure the safety of food products against foodborne pathogens such as *Salmonella*.

2.7: Current *Salmonella* Control Methods for Raw Poultry Products

Adequate cooking of raw poultry products to 165°F ensures the elimination of *Salmonella* according to the USDA. It is important for consumers in regards to raw poultry products to cook to an internal temperature of 165°F as it will eliminate all foodborne pathogens present in the product regardless of time. Proper cooking of raw poultry by the consumer and poultry processor for fully-cooked products sold at retail can help to prevent foodborne illness. Reduction or elimination of foodborne pathogens in raw poultry products prior to cooking can help to reduce risk of foodborne illness. The food industry has put in place some control measures for raw meat products to increase shelf-life and reduce risk of foodborne illness such as application of antimicrobials, modified atmospheric packaging (MAP) and refrigeration. Additionally, non-thermal post lethality

treatments such as irradiation and high pressure processing (HPP) are emerging to eliminate the threat of post-process contamination of a cooked product and further ensure the safety of the product prior to consumption.

Peracetic acid (PAA) is one of the main antimicrobial treatments commonly used post-slaughter to reduce levels of *Salmonella* present on the meat. It is mostly used to treat wastewater in the poultry industry as it is considered to be a strong disinfectant. PAA increases in antimicrobial activity with increasing temperature and decreasing pH. One study showed that PAA used at a concentration of 0.0025% showed a 4log CFU/g reduction of *Salmonella* on poultry carcasses after 1h exposure at 4°C in chilled water (Bauermeister et al. 2008). The maximum concentration of PAA to be in contact with poultry in water is 0.2% (FDA 2014). PAA shows to be an effective control method in reducing *Salmonella* levels on poultry carcasses prior to further processing.

MAP is a common method used to improve the shelf life of fresh chicken meat utilizing different compositions of nitrogen, CO_2, and oxygen. It has been found in studies that MAP compositions containing elevated CO_2 is more effective at controlling *Salmonella* growth in chicken meat (Nychas and Tassou 1996). In addition, MAP with (70% CO_2/30% N_2) was found to reduce *Enterobacteriaceae* population approximately 2 log CFU/g more than MAP with (30%CO_2/70% N_2) (Chouliara et al. 2007). It has also been shown that MAP combined with oregano essential oil is able to extend the shelf life of meat an extra 1-2days compared to MAP by itself (Chouliara et al. 2007). This shelf life extension by MAP not only helps to keep the meat on the market longer but also reduces level of potential foodborne pathogens that may be present on the product. The application

of combining MAP with essential oils shows promise in effectively reducing levels of foodborne pathogens present in the product leading to a reduced risk of foodborne illness.

Irradiation is a method that is not widely used in the meat industry but is effective at reducing pathogen and spoilage microorganisms. Main reason for this low usage is due to low consumer acceptability. Although, irradiation of chicken meat has been approved as safe to use by the FDA (FDA 2016[a]). Additionally, irradiation has proven to be an effective method in reducing levels of foodborne pathogens that could be present in the product as well as natural microflora (Silva et al. 2016). Currently the standards set by the FDA for maximum dosage of irradiation for uncooked refrigerated and frozen poultry products are 4.5 kilograys (kGy) and 7.0 kGy respectively (FDA 2016[b]).

EO's could potentially be used in direct application of the poultry surface and interior of the meat to reduce and prevent *Salmonella* from reaching infectious doses. Additionally, most of these compounds mentioned such as carvacrol, thymol, and WMEO have shown strong antimicrobial activity against *Salmonella* (Helander et al. 1998; Monu et al. 2014; Nazer et al. 2005; Olasupo et al. 2003).The use of these antimicrobials could also delay growth of *Salmonella* in temperature abuse conditions allowing longer preservation of the meat and a reduced risk of foodborne illness.

Chapter 3: Materials and Methods:

3.1: Culture preparation and storage

Salmonella Enteritidis, Typhimurium, Heidelberg, Hartford, Senftenberg, Orion, Kentucky and Montevideo (obtained from Nelson Cox, USDA-ARS, Athens, Georgia) were used in this study. Frozen cultures of each serovars were grown in 9 mL tryptic soy broth (TSB) with an initial pH of 7.2 (Neogen, MI) at 37 °C for 20-24h. Cultures were transferred separately into another sterile 9 mL tryptic soy broth tube and incubated at 37 °C for 20-24h. All *Salmonella* serovars were then streaked on tryptic soy agar (TSA) (Neogen, MI) and XLT4 (Difco) media and incubated in the same conditions mentioned previously to ensure that cultures were pure. After incubation, TSA plates were sealed using parafilm, stored at 4 °C and re-streaked every 3 weeks to ensure viability. Prior to experiments, inoculum levels of each organism were verified in triplicate by serially diluting and spread plating overnight cultures on TSA to determine the CFU/mL after 20h of incubation at 37 °C.

Salmonella Enteritidis, Typhimurium, Heidelberg, Hartford, Senftenberg and Orion were grown overnight at 37°C for 20-24h in TSB and subcultured twice. For, each experiment, cultures were grown overnight at 37°C for 20-24h and transferred again to TSB using the same conditions.

3.2: Macrodilution assay testing of WMEO against *Salmonella*

Salmonella cultures were diluted in TSB and 20 uL was added to 9.8 mL of TSB broth to achieve an inoculum level of approximately 10^5 CFU/mL. A 6-serovar cocktail was made using the serovars mentioned previously. The cocktail was made by serially

diluting each serovars in TSB and combining 20uL of each strain into 9.7mL TSB to achieve a final concentration of *Salmonella* of approximately 10^5 CFU/mL.

Preparation of WMEO solutions were made by diluting the stock solution of crude WMEO (containing 12% 4-HBITC Procter & Gamble, Mason, OH) with dimethyl sulfoxide (DMSO) (Macron Fine Chemicals, Center Valley, PA) which allowed the WMEO to be dispersed into an aqueous solution. Final concentrations of WMEO solutions were obtained by adding 0.2mL of each diluted stock solution in the inoculated 9.7mL and 9.8mL TSB tube. Each serovar of *Salmonella* and the cocktail were exposed to final concentrations of 0.84%, 0.42%, and 0.21% WMEO or 0.1% 4-HBITC, 0.05% 4-HBITC, and 0.025% 4-HBITC, respectively. A positive control had no treatment and instead 0.2mL of 100% DMSO was added to the 9.8mL TSB tube giving a final concentration of 2% DMSO.

After inoculum and antimicrobial were added, assay tubes were stored at 22°C for 24h. The effect of each treatment was measured by serially diluting in 0.1% peptone water (PW) (Neogen, MI) and spread plating on TSA. Plates were incubated at 37°C for 24h before counting colonies. Each treatment was performed in duplicate and all experiments were repeated in triplicate.

3.3: MIC determination of WMEO against *S*. Typhimurium

The macrodilution assay was used instead of the microdilution method to determine the MIC of WMEO against *S*. Typhimurium due to turbidity interfering with optical density readings to determine microbial growth. The culture was prepared as previously described and 20 µL was added to 9.8 mL of TSB.

WMEO concentrations were prepared from the same stock solution mentioned previously using DMSO. Diluted antimicrobial concentrations consisted of 27.2%, 25%, 24.6%, 24% and 23% WMEO. Final concentrations in assay tube were prepared by taking 0.2 mL of each diluted concentration and adding each one separately into an inoculated 9.8 mL TSB tube. The positive control had no treatment and instead 0.2 mL of 100% DMSO was added to the 9.8 mL TSB tube giving a final concentration of 2% DMSO.

Samples were stored at 22°C for 48h. Serial dilutions of each treatment in 0.1% PW were spread plated on TSA at 24h and 48h. Plates were incubated at 37°C for 24h before counting colonies. Each treatment was performed in duplicate and the experiment was repeated in triplicate.

3.4: MIC determination of carvacrol and thymol against *S*. Typhimurium

Overnight *Salmonella* cultures were prepared as previously described and serially diluted in TSB. The microdilution method was used to assess the antimicrobial activity of carvacrol and thymol in 96-well plates. Aliquots of 120 µL TSB were added to each well labeled 1-7 and 9 and inoculum was then added in 30uL aliquots into the same wells. Column 9 consisted of the positive control.

Pure solutions of carvacrol (Tokyo Chemical Industry, Tokyo, Japan >98.0%) and thymol (Acros Organics, NJ, USA >99%) were used to prepare 10% stock solutions in DMSO. Stock solutions were further diluted using 10% DMSO to concentrations of 1% carvacrol and thymol. Stock solutions were again diluted with TSB to obtain the following concentrations: 0.0625%, 0.0563%, 0.05%, 0.0438%, 0.0375%, 0.0313%,

0.0250%, 0.0188%, and 0.0125%. Final concentrations in the microtiter plate were

acquired by taking 0.1mL of each concentration mentioned previously to obtain 0.0250%,

0.0225%, 0.0200%, 0.0175%, 0.0150%, 0.0125% and 0.0100% of carvacrol in wells 1-7,

respectively. The same procedure was carried out for thymol. The negative control

consisted of 150uL of TSB and 100uL of DMSO in row E columns 1-7. Microtiter plates

were incubated at 22°C for 48h.

OD measurements were taken at 595nm at 0h, 24h and 48h. MIC was defined as

the well with the lowest concentration of antimicrobial that showed no growth: an OD

change less than 0.05 after 48h incubation. Experiment was repeated in triplicate.

3.5: Antimicrobial effect of combining WMEO with carvacrol or thymol

In this test, the macrodilution assay was used against *S*. Typhimurium. The

culture was prepared as previously described and 20uL was added to 9.8mL of TSB.

MIC's of WMEO, carvacrol and thymol were determined in the previous study to be

0.5%, 0.02% and 0.02%, respectively. Binary combinations of the antimicrobials were

used as treatments based on the method of Techathuvanan et al. (2014) to evaluate if the

combined antimicrobials exhibited a synergistic, additive, or antagonistic effect. These

effects were determined by the fractional inhibitory concentration index as <0.5, 1, and

>1.5 which equals to synergistic, additive and antagonistic effect, respectively.

Synergistic, additive and antagonistic effect is the result of combined antimicrobials

having an improved effect, the same effect and a reduced effect respectively, compared to

the MIC of the compounds alone. Stock solutions of WMEO, carvacrol and thymol were

diluted with DMSO to make the following binary combinations: 0.5 WMEO MIC + 0.5

Carvacrol MIC, 0.75 WMEO MIC + 0.75 Carvacrol MIC, 0.75 WMEO MIC + 0.25

Carvacrol MIC and 0.25 WMEO MIC + 0.75 Carvacrol MIC. Thymol was combined

with WMEO in the same concentrations as carvacrol. The positive control had 2%

DMSO present in solution instead of the treatment. In addition, WMEO, carvacrol and

thymol MIC's alone were tested again in this experiment for further verification.

Treatments were applied in duplicate and the experiment was repeated in triplicate.

Table 3.5.1 Legend key for figure 4.3.1

Abbreviation	Concentration of antimicrobials used
positive	positive
Treatment 1	MIC WMEO
Treatment 2	MIC Carvacrol
Treatment 3	MIC Thymol
Treatment 4	0.5% MIC WMEO and Carvacrol
Treatment 5	0.5% MIC WMEO and Thymol
Treatment 6	0.75% MIC WMEO and Carvacrol
Treatment 7	0.75% MIC WMEO and Thymol
Treatment 8	0.75% MIC WMEO and 0.25% MIC Carvacrol
Treatment 9	0.75% MIC WMEO and 0.25% MIC Thymol
Treatment 10	0.25% MIC WMEO and 0.75% MIC Carvacrol
Treatment 11	0.25% MIC WMEO and 0.75% MIC Thymol

3.6: Application of WMEO and carvacrol to ground chicken at 4 °C and 10 °C

In this experiment, nalidixic acid (NAL) resistant (up to 100ppm), serovars of *S.*

Enteritidis, Typhimurium, Heidelberg, Kentucky and Montevideo were used in a 5-

serovar cocktail. The bacterial population of overnight cultures of each serovar were

determined in 100ppm NAL TSB before performing the experiment. The cocktail was

prepared by first washing cultures by centrifuging 1.5mL of overnight cultures in

microcentrifuge tubes at 9,391x *g* for 2 minutes (Eppendorf centrifuge 5424R,

Hauppauge, NY). The supernatant was removed and replaced with 1.5mL of 0.1% PW.

Tubes were vortexed vigorously before centrifuging again. Cultures were washed again before preparing the cocktail. In preparation of cocktail, 136.8mL of 0.1% PW was dispensed in a sterile flask with sealed lid. Washed cultures of *S.* Typhimurium, Heidelberg, Montevideo, Kentucky, and Enteritidis were dispensed in the flask with the following volumes respectively: 0.16 mL, 0.21 mL, 0.36 mL, 0.58 mL and 1.94 mL to achieve a final bacterial load of approximately (10^7 CFU/mL).

Ground chicken consisted of boneless chicken thighs with skin from the Auburn University Poultry Science Research Unit. Chicken thighs were ground using Mini-32 Biro grinder (MFG Co. Marblehead, Ohio) and placed into aluminum trays. The positive and negative controls both consisted of 2100g and 1800g of ground chicken for the 4 °C and 10 °C trials respectively. As for the treatments, 1800g and 1500g of ground chicken were placed in aluminum trays for 4 °C and 10 °C trials respectively. Meat was inoculated by adding 1mL of cocktail per 100g of chicken and mixing in aluminum trays by hand. After mixing, aluminum trays were stored in the refrigerator at 4 °C for 30 minutes to allow for bacterial attachment.

There were five treatments applied to the ground chicken in addition to the positive and negative control. Antimicrobial treatments applied were as follows: 0.75% WMEO, 0.5% WMEO, 0.75% + 0.1% carvacrol, 0.5% WMEO + 0.1% carvacrol and 0.1% carvacrol. Antimicrobial solutions were made by diluting WMEO and carvacrol stock solutions with propylene glycol (Amresco, LLC, OH) on the day of the experiment. For 4 °C and 10 °C, 30 mL and 36 mL, respectively of each antimicrobial solution were added to the ground chicken to achieve the previously mentioned concentrations. Positive

and negative controls both had 42 mL and 36 mL of propylene glycol added to the meat

in order to ensure that the addition of this substance had no effect on the growth of

Salmonella for 4 °C and 10 °C trials, respectively. In addition, to account for the moisture

added by the inoculum in the treatments, 21mL and 18mL of 0.1% PW were added to the

negative control for the 4 °C and 10 °C trials, respectively. Individual samples were made

on the first day of each trial by taking 100g from each treatment batch and sealing with

polyethylene film in styrofoam trays (Genpak, Glens Falls, NY) with Driloc DLSA 100

5.5''x7.0'' absorbent pads (Novipak Reading, PA).

The 4 °C trial was monitored for 12 days sampling every 2 days by taking 25g of

ground chicken from triplicate trays and adding it to a stomacher bag filled with 225mL

of buffered peptone water (BPW) (Criterion) and stomached at 230 rpm for 2 minutes.

After stomaching, 10-fold serial dilutions were made using BPW and plated on 100 ppm

NAL TSA (Criterion). Plates were incubated at 37 °C for 24 h before colonies were

counted and recorded. Sampling procedure was the same for the 10 °C trial, but with an 8

day storage period. In addition, sampling was done every 2 days with additional sampling

on the 7th day. A *Salmonella* enrichment method according to USDA were performed on

negative samples on Day 0 to verify that the chicken used was free of *Salmonella* (USDA

2017). Ground chicken was stomached with 225 mL of BPW and incubated at 35±2 °C

for 20-24 h according to MLG 4.09 procedure 4.5.2 (USDA 2017). After incubation

0.5mL of each bag were dispensed in 10 mL of tetrathionate broth (TT) and incubated at

42±0.5 °C for 22-24 h in accordance to procedure 4.6 in the MLG 4.09 by USDA.

Samples were then streaked on XLT4 media for identification of *Salmonella* (USDA

2017). Each treatment was conducted in triplicate and experiments were performed 3 times.

3.7: pH, Moisture and Fat Analysis of Ground Chicken

In addition to microbial sampling, pH was measured for each of the treatments over both storage periods at 4 °C and 10 °C. The pH meter was calibrated prior to taking measurements. Measurements were performed on each tray using a fisher scientific accumet xl15 resulting in three pH values for each treatment per sampling day.

The forced air dry oven method, AOAC 950.46B was used to analyze the moisture content of ground chicken for each trial. Approximately, 5g of sample was weighed out and dried at 95-105°C for 16-18h in replicates of three. Dried samples were then placed into a desiccator to allow to cool for 1h. After cooling weight was recorded. Dried samples were then analyzed for crude fat using AOAC 960.39 via Goldfisch method. Approximately, 2-3g of dried chicken were placed in a thimble in replicates of three. Fat was extracted using 50mL of petroleum ether (Macron Fine Chemicals) in a beaker.

3.8: Statistical Analysis

Statistical analysis was performed using SAS 9.4 (SAS Institute Inc., Cary, NC). Analysis of variance (ANOVA) was performed using the Student-Newman Keuls (SNK) test to compare the means at a confidence interval of 95%.

Chapter 4: Results and Discussion

4.1 Effect of WMEO against different serovars of *Salmonella enterica*

The growth of *Salmonella enterica* serovars was evaluated in the presence of WMEO in TSB at 22°C. WMEO was effective against *S.* Typhimurium, Enteritidis, Heidelberg, Hartford, Senftenberg and Orion and no differences in level of resistance among the serovars were found statistically. However, at 0.21%, WMEO had a greater effect against *S.* Enteritidis after 24 h compared to the other serovars (Figure 4.1.1). Additionally, SNK test showed that the control, 0.84%, 0.42% and 0.21% WMEO were statistically different from each other (Figure 4.1.1).WMEO at 0.84%, 0.42% and 0.21% compared to the positive control showed approximately a 6-7 log, 3-4 log and 1 log difference for all serovars respectively. Final levels of *Salmonella* were lower than at 0h exposure at 0.84% WMEO. Additionally, no significant increase in growth occurred at 0.42% WMEO. Further research was conducted to investigate the efficacy of WMEO in a cocktail (Figure 4.1.2) at the same inoculum level (10^5 CFU/mL) as tested previously. It was found that WMEO had the same effect against the serovars individually as it did against the cocktail.

Table 4.1.1: Antimicrobial effect of WMEO against *Salmonella* at 22 °C in TSB after 24h

Serovar	Control	0.21% WMEO	0.42% WMEO	0.84% WMEO
		Treatment		
Enteritidis	8.96 ± 0.13^A	7.44 ± 0.45^B	4.81 ± 0.22^A	3.07 ± 0.97^A
Typhimurium	8.46 ± 0.07^B	8.08 ± 0.79^{AB}	4.53 ± 0.64^A	1.37 ± 0.49^A
Orion	8.90 ± 0.10^A	8.15 ± 0.32^{AB}	5.14 ± 1.11^A	1.28 ± 0.43^A
Heidelberg	8.98 ± 0.06^A	8.61 ± 0.17^A	4.34 ± 0.75^A	1.82 ± 0.83^A
Senftenberg	8.97 ± 0.01^A	8.66 ± 0.09^A	4.88 ± 1.46^A	1.00 ± 0.17^A
Hartford	8.87 ± 0.06^A	8.64 ± 0.10^A	5.30 ± 0.39^A	2.55 ± 1.42^A

Values are mean ± standard deviation
Within each treatment values with different letters are significantly different ($p<0.05$)

Figure 4.1.1: Antimicrobial effect of WMEO against *Salmonella* serovars at 22°C in TSB after 24h exposure
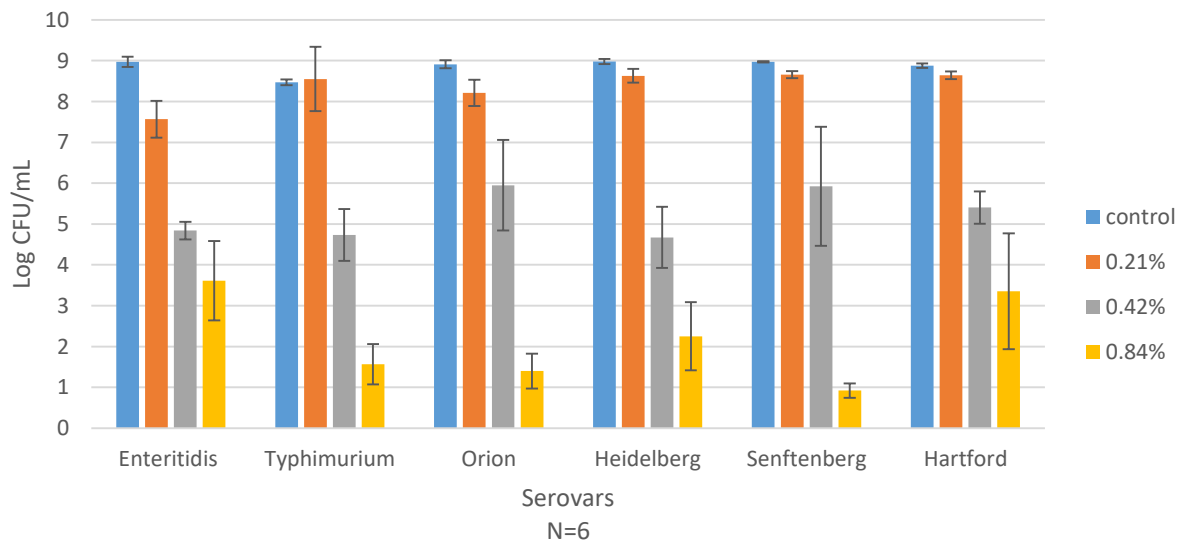
Table 4.1.2: Antimicrobial effect of WMEO against a cocktail of *Salmonella* serovars at 22°C in TSB after 24h

	Treatment			
	control	0.21% WMEO	0.42% WMEO	0.84% WMEO
Time				
0h	5.18 ± 0.21^a	5.08 ± 0.11^a	5.03 ± 0.04^a	4.81 ± 0.15
24h	9.27 ± 0.06^a	8.42 ± 0.19^a	4.51 ± 0.78^b	2.28 ± 1.16^c

Values are means log CFU/mL ± standard deviation
Within each column and row values with different letter grouping are significantly different (p<0.05).
N=6

Figure 4.1.2: Antimicrobial effect of WMEO against a cocktail of *Salmonella* serovars

incubated in TSB at 22 °C after 24h
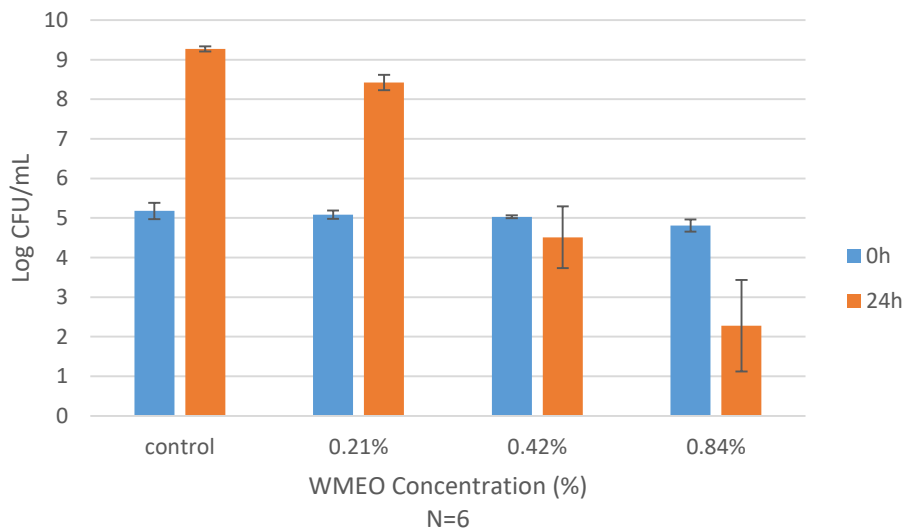
4.2 MIC Determination of WMEO, Carvacrol and Thymol

Before conducting a synergism study, MIC's of the individual antimicrobials needed to be established. In this study, MIC was defined as the applied concentration of antimicrobial at which the target organism did not show growth of more than 1 log after 48h exposure (Helander et al. 1998; Nazer et al. 2005; Olasupo et al. 2003). The results showed that WMEO had a MIC of 0.5% at 22°C against *S*. Typhimurium using the macrodilution method. After 48 h a 1 log reduction was seen at a concentration of 0.5% WMEO. In comparison to other literature, the MIC against *S*. Enteritidis was established to be 0.15% WMEO (Techathuvanan et al. 2014). These differences between the current study and literature in MIC against *Salmonella* may be attributed to media pH. The tryptic soy broth used in the previously mentioned study had an initial pH of 6.0 compared to an initial pH of 7.2 used in this study for TSB. Allyl isothiocyanate (AIT), another isothiocyanate, has shown increased activity in vitro at lower pHs. One study investigating the effect of pH on the activity of AITC against *Salmonella* found that 200 ppm AITC showed more inhibition of *Salmonella* at a pH of 5 than at a pH of 7 at 21°C in Mueller-Hinton broth (MHB) (Olaimat et al. 2013); *Salmonella* at a pH of 5 showed a 3 logs difference compared to pH 7. Another study reported that the MIC of AIT for *E. coli* O157:H7 was 50ppm at pH 6.5 in TSB. However, when tested at a pH of 7.5 the MIC changed to 250 ppm AIT (Luciano et al. 2009). These results suggest that isothiocyanates exhibit stronger antimicrobial activity at lower pHs. Additionally, improved antimicrobial action has been shown with other essential oils at lower pH values (Burt 2004; Juven et al. 1994; Skandamis et al. 2000).

34

The OD$_{595}$ was measured at the concentrations of carvacrol and thymol tested (data not shown) and it was determined that the MICs were 0.02% as the change in OD was < 0.05 at these concentrations. These results are the same as what other studies have found (Nazer et al. 2005, Olasupo et al. 2003, Helander et al. 1998).

4.3 Antimicrobial effect of combining WMEO with carvacrol or thymol

Antimicrobial binary proportions were based off the fractional inhibitory concentration indexes (FICI) used by Techathuvanan et al. (2014). In the binary combination of 0.75% MIC of WMEO and 0.75% MIC carvacrol there was a 1.5 log reduction of *S.* Typhimurium after 48h (Figure 4.3.1). This absence of growth indicates that carvacrol had an additive effect when combined with WMEO. The same additive effect was seen when thymol and WMEO were combined against *S.* Typhimurium. All other fractional combinations resulted in growth of the organism after 48h exposure which is shown in Figure 4.3.1. Techathuvanan et al (2014) also found that the compounds lauric arginate (LAE) and citrus flavonoid and acid blend (CFAB) are antimicrobial compounds that had additive effects with WMEO against *S.* Enteritidis (Techathuvanan et al. 2014). However, olive pulp extract (OE) combined with WMEO achieved a synergistic effect against *S.* Enteritidis (Techathuvanan et al. 2014). The additive and synergistic effects achieved from combining WMEO with the compounds mentioned previously indicate potential of these compounds to be used as antimicrobial solutions to control *Salmonella* in a food product. It is known that higher concentrations of EO's are needed to achieve inhibitory effects against microorganisms in a food product due to a reduced efficacy of antimicrobial activity via interaction of food components such as fat and starch (Gutierrez

et al. 2008, Cava et al. 2007, Tassou et al. 1995). These combinations can help to reduce

the concentration needed to achieve an inhibitory or bacteriostatic effect on *Salmonella* in

a food product which would also reduce the cost and negative sensory effects.

Table 4.3.1 Design of binary combination tests with fractional inhibitory concentration

index (FICI)

A	B	FICI	Bacterial growth result	Effect[a]
Binary Antimicrobial combinations				
0	0	0	Growth	Control
1	0	1	No growth	MIC
0	1	1	No growth	MIC
3/4	1/4	1	Growth	Additive or antagonistic
			No growth	Additive
1/2	1/2	1	Growth	Additive or antagonistic
			No growth	Additive
1/4	3/4	1	Growth	Additive or antagonistic
			No growth	Additive
1/4	1/4	0.5	Growth	Additive or antagonistic
			No growth	Synergistic
3/4	3/4	1.5	Growth	Antagonistic
			No growth	Additive or synergistic

[a]Synergistic, additive and antagonistic correspond to FICIs of <0.5, 1.0 and >1.5, respectively.
(Adapted from Techathuvanan et al. 2014)

Figure 4.3.1: *S.* Typhimurium exposed to various concentrations of WMEO, carvacrol and thymol for 48 h at 22 ºC
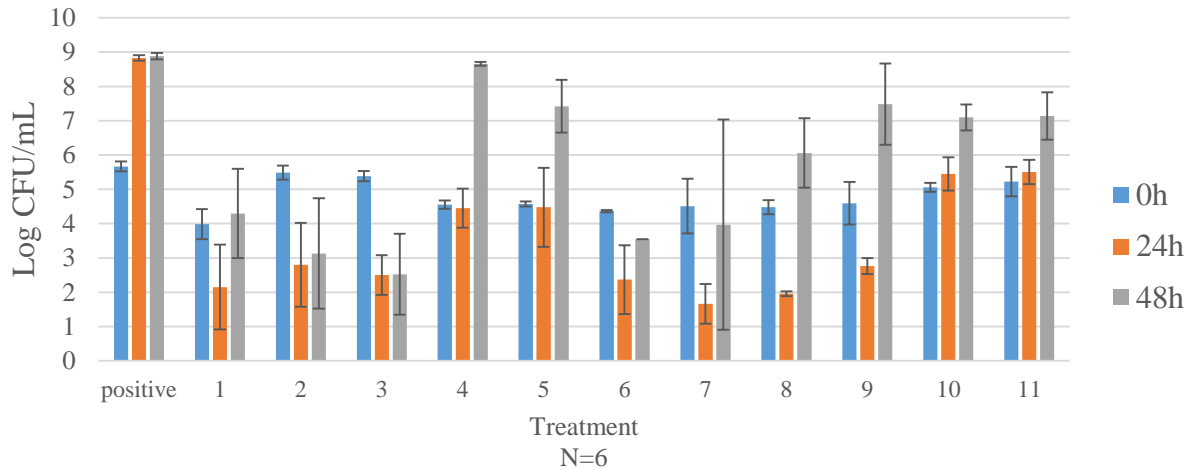
4.4 Antimicrobial activity of WMEO and carvacrol against *Salmonella* in ground chicken

Enrichment of *Salmonella* was done on the negative control using USDA method resulting in no detection of the organism. Sampling of the ground chicken was continued for the negative control, plating on TSA with 100ppm NAL resulting in no growth on the plate with a detection limit of < 2 log CFU/g. Table 4.4.1 shows log values of *Salmonella* in chicken over a 12 day period in the presence of different concentrations of WMEO and carvacrol. All treatments, with the exception of 0.1% carvacrol, were significantly different (p<0.05) from the positive control throughout 12 days of storage. At Day 10 and 12, 0.75% WMEO + 0.1% carvacrol showed 0.6 log CFU/g less counts of *Salmonella* compared to the positive control. For all other treatments, approximately 0.5 log CFU/g difference was seen after 12 days of storage compared to the positive control with the

37

exception of 0.1% carvacrol which had no effect. The combination of WMEO and carvacrol shows potential in being used together as it lowered the concentrations of *Salmonella* compared to the positive control. In Figure 4.4.1, it can be seen that the positive control initially had a slight decrease in population after 2 days of storage. However, the population of *Salmonella* does not change over the next 10 days of storage. The results from this study is further confirmed by previous studies that have shown *Salmonella* growth to be static at temperatures of 4 °C and below in chicken breasts and thighs (Pintar et al. 2007). The initial decrease in population suggests *Salmonella* adaptation to refrigeration temperature.

Figure 4.4.1: Antimicrobial effect of WMEO and carvacrol on *Salmonell*a in ground chicken at 4 °C
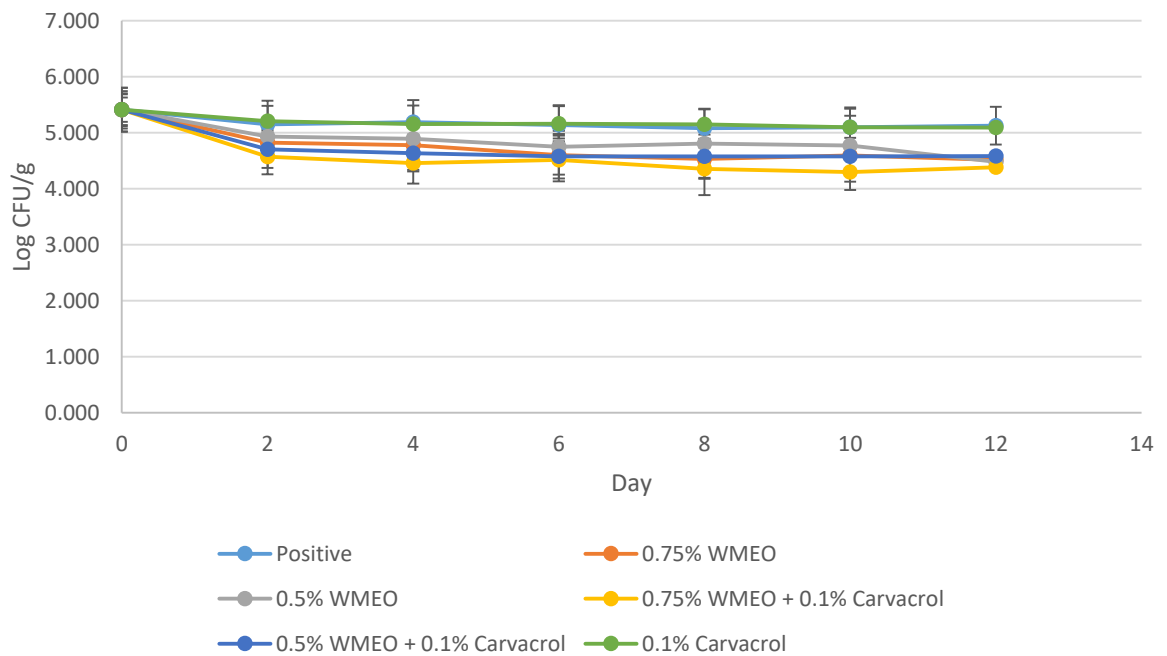
Figure 4.4.2 shows the effect of WMEO and carvacrol over an 8 day storage period at 10 °C. Results show that at the highest concentration of antimicrobial (0.75% WMEO + 0.1% carvacrol) a bacteriostatic affect was observed up to the 7th day of storage at temperature abuse of 10 °C. After 8 days of storage at 10 °C a 3 log CFU/g increase in *Salmonella* was observed in the positive control. The behavior of *Salmonella* growth in this study is similar to previous studies which a 2-3 log CFU/g increase has been seen with *Salmonella* at temperature abuse of 10 °C in chicken thighs and breast (Pintar et al. 2007). As observed in table 4.4.2, 0.75% WMEO, 0.5% WMEO + 0.1% carvacrol, and 0.75% WMEO + 0.1% carvacrol were statistically different (p<0.05) than 0.5% WMEO after 2 days of storage. Although, over the course of the 8 days, 0.75% WMEO + 0.1% carvacrol showed to be the most effective treatment against *Salmonella* and statistically different than all other treatments on day 2, 4, 6 and 8. In addition, at the final day of storage all treatments were approximately 1 log CFU/g less than the positive control and 0.1% carvacrol.

Figure 4.4.2: Antimicrobial effect of WMEO and carvacrol on *Salmonella* in ground chicken at 10 °C
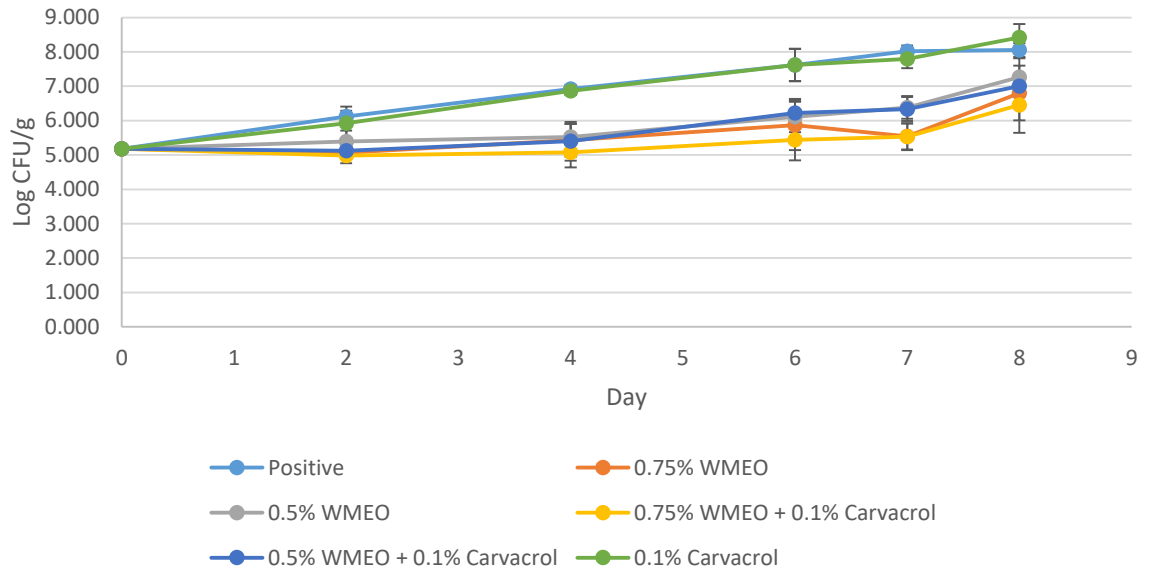
Table 4.4.1: Antimicrobial effect of WMEO and carvacrol on *Salmonella* spp. in ground chicken at 4°C

	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
Positive	5.41 ± 0.33^A	5.15 ± 0.33^A	5.19 ± 0.40^A	5.14 ± 0.35^A	5.08 ± 0.34^A	5.10 ± 0.34^A	5.13 ± 0.34^A
0.75% WMEO		4.83 ± 0.28^C	4.78 ± 0.24^C	4.60 ± 0.44^C	4.53 ± 0.35^C	4.60 ± 0.36^C	4.52 ± 0.31^{BC}
0.5% WMEO		4.93 ± 0.22^B	4.90 ± 0.25^B	4.75 ± 0.35^B	4.81 ± 0.28^B	4.78 ± 0.35^B	4.48 ± 0.53^B
0.75% WMEO + 0.1% Carvacrol		4.58 ± 0.39^E	4.46 ± 0.31^E	4.52 ± 0.37^D	4.36 ± 0.39^D	4.30 ± 0.47^D	4.39 ± 0.32^C
0.5% WMEO + 0.1% Carvacrol		4.71 ± 0.40^D	4.64 ± 0.33^D	4.58 ± 0.33^C	4.58 ± 0.40^C	4.58 ± 0.39^C	4.59 ± 0.45^{BC}
0.1% Carvacrol		5.21 ± 0.30^A	5.16 ± 0.36^A	5.16 ± 0.33^A	5.15 ± 0.32^A	5.10 ± 0.28^A	5.09 ± 0.35^A

Values are mean log CFU/g ± standard deviation;
Within each day values with different letters are significantly different ($p<0.05$)
N=9

Table 4.4.2: Antimicrobial effect of WMEO and carvacrol on *Salmonella* spp. in ground chicken at 10 °C

	Day 0	Day 2	Day 4	Day 6	Day 7	Day 8
Positive	5.18 ± 0.11^A	6.12 ± 0.16^A	6.92 ± 0.12^A	7.62 ± 0.46^A	8.02 ± 0.16^A	8.05 ± 0.20^B
0.75% WMEO		5.07 ± 0.14^D	5.43 ± 0.48^B	5.87 ± 0.73^C	5.54 ± 0.38^D	6.80 ± 0.80^D
0.5% WMEO		5.39 ± 0.32^C	5.53 ± 0.38^B	6.11 ± 0.44^{BC}	6.38 ± 0.33^C	7.26 ± 0.55^C
0.75% WMEO + 0.1% Carvacrol		4.98 ± 0.22^D	5.08 ± 0.44^C	5.44 ± 0.59^D	5.53 ± 0.39^D	6.46 ± 0.81^E
0.5% WMEO + 0.1% Carvacrol		5.12 ± 0.15^D	5.40 ± 0.56^B	6.22 ± 0.41^B	6.34 ± 0.35^C	7.00 ± 0.30^{CD}
0.1% Carvacrol		5.92 ± 0.48^B	6.87 ± 0.13^A	7.62 ± 0.47^A	7.79 ± 0.27^B	8.42 ± 0.39^A

Values are mean log CFU/g ± standard deviation
Within each day values with different letters are significantly different ($p<0.05$)
N=9

4.5 Fat, Moisture and pH of ground chicken

Fat content was analyzed as fat is one major food component that greatly reduces the efficacy of essential oil activity in addition to high moisture content (Gutierrez et al. 2008).The average moisture and fat content for the ground chicken thighs with skin used at 4°C and 10°C were 68.6% moisture and 12.8% fat and 69.0% moisture and 11.9% fat, respectively. As for pH, a range of 6.4-6.9 was seen for ground chicken thighs throughout a storage period of 8 and 12 days at 10°C and 4°C, respectively. One study observed a pH range of 6.6-6.7 for a 7 day storage period at 4°C for ground chicken thighs (Sharma et al. 2013). These results are similar to what was found in this experiment at 4°C. The ground chicken samples treated with antimicrobials in the 10°C experiment showed a pH increase of 0.1 on day 0 compared to the negative control. Similarly, the positive control showed a pH of 6.5 which was also higher than the observed pH in the negative control samples on day 0. The pH of the ground chicken samples increased between day 4 and day 6. The pH increased to final reading of 6.7 for the positive control and all treatments at 10°C.

Table 4.5.1: pH values of ground chicken at 4°C

	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
un-inoculated	6.71±0.01	6.70±0.15	6.47±0.10	6.59±0.15	6.68±0.20	6.60±0.18	6.67±0.18
positive	6.73±0.03	6.68±0.18	6.48±0.15	6.58±0.08	6.53±0.20	6.69±0.16	6.86±0.21
0.75% WMEO		6.84±0.10	6.40±0.17	6.68±0.10	6.48±0.19	6.65±0.12	6.63±0.24
0.5% WMEO		6.82±0.09	6.46±0.22	6.65±0.13	6.57±0.23	6.59±0.19	6.71±0.24
0.75%WMEO+0.1% Carvacrol		6.78±0.14	6.45±0.17	6.64±0.13	6.54±0.20	6.52±0.20	6.67±0.30
0.5% WMEO +0.1% Carvacrol		6.72±0.13	6.53±0.25	6.61±0.12	6.64±0.14	6.52±0.32	6.67±0.27
0.1% Carvacrol		6.79±0.11	6.54±0.18	6.62±0.09	6.60±0.12	6.78±0.08	6.69±0.31

*Values are mean ± standard deviation

Table 4.5.2: pH values of ground chicken at 10°C

	Day 0	Day 2	Day 4	Day 6	Day 7	Day 8
un-inoculated	6.42±0.14	6.43±0.12	6.47±0.11	6.59±0.15	6.58±0.11	6.60±0.10
positive	6.51±0.06	6.52±0.14	6.60±0.16	6.54±0.10	6.71±0.07	6.73±0.09
0.75% WMEO	6.57±0.13	6.51±0.15	6.57±0.09	6.56±0.08	6.58±0.07	6.67±0.06
0.5% WMEO	6.55±0.06	6.65±0.11	6.50±0.17	6.60±0.15	6.51±0.08	6.69±0.13
0.75%WMEO+0.1% Carvacrol	6.50±0.03	6.54±0.16	6.52±0.10	6.57±0.08	6.60±0.11	6.71±0.16
0.5% WMEO +0.1% Carvacrol	6.49±0.09	6.60±0.17	6.57±0.14	6.62±0.11	6.59±0.10	6.67±0.09
0.1% Carvacrol	6.49±0.20	6.59±0.10	6.60±0.16	6.66±0.17	6.70±0.04	6.71±0.12

*Values are mean ± standard deviation

Chapter 5: Conclusions

The purpose of this study was to evaluate the antimicrobial activity of WMEO against *Salmonella enterica* serovars Enteritidis, Typhimurium, Heidelberg, Senftenberg, Orion, Hartford, Kentucky and Montevideo and investigate synergistic activity with other antimicrobials. WMEO was also applied to ground chicken to see its effectiveness at controlling *Salmonella* in a food product during storage at 4°C and 10°C.

It was found that WMEO had equal effectiveness at inhibiting all *Salmonella* serovars. In addition, no difference in log reductions were found when applying WMEO to a 6-strain cocktail versus testing the serovars individually. These results lead us to conclude that WMEO proposes to be an effective natural antimicrobial to be used against *Salmonella* as all serovars had equal susceptibility to WMEO. In addition, it was found that the MIC of *S*. Typhimurium was 0.5%.

A synergism study showed that WMEO in combination with carvacrol or thymol had an additive effect. After 48h exposure only the 0.378% WMEO + 0.015% carvacrol and 0.015% thymol showed no growth of *S*. Typhimurium. As carvacrol exhibited more consistent results than thymol when combined with WMEO, that combination was applied to ground chicken.   The application of 0.75% WMEO + 0.1% carvacrol in ground chicken at 4 °C showed a 0.7 log CFU/g reduction after 12 days of storage while all other treatments except the positive control and 0.1% carvacrol, showed a 0.5 log CFU/g reduction. However, results indicate that the combination slowly inhibited *Salmonella* overtime. The log population of *Salmonella* slowly decreased each day for all treatments up to Day 10 at 4 °C. This shows that the WMEO and carvacrol did not lose

activity during the experiment. Additionally, the refrigeration temperature had a bacteriostatic effect allowing the antimicrobial to put more stress on the organism to delay exponential growth and reduce levels up to 1log CFU/g. *Salmonell*a in ground chicken stored at abusive temperatures behaved differently. After 2 days, *Salmonella* grew approximately 1 log CFU/g at 10 °C for the positive control and 0.1% carvacrol. In comparison to other treatments at 10 °C no growth was observed after 2 days of storage. The growth of *Salmonella* was controlled for 7 days using 0.75% +0.1% carvacrol treatment. At the final day of storage, all treatments containing WMEO had significantly lower levels of *Salmonella* than the control, and treatments with 0.75% WMEO were ca. 2.5 log CFU/g lower than the control. The addition of 0.1% carvacrol did not appear to enhance the efficacy of WMEO. The efficacy of the antimicrobials were apparently reduced when applied to chicken as compared to *in vitro* studies. One reason for this decrease is most likely due to the hydrophobic nature of these antimicrobials allowing them to dissolve into the lipid portion of the chicken, while the bacteria remained in the water phase. This mechanism would propose that WMEO and carvacrol only interacted with a certain population and explain why lower reductions were seen, as *Salmonella* populations could be present in both the lipid and aqueous phases of the ground chicken.

In conclusion, WMEO shows potential as a natural preservative in controlling *Salmonella* in chicken products. However, further research is needed to investigate the toxicity of the oil and as well as investigate the most efficient way to apply it to a food product.

References

1. Anand SP, Sati N. 2013. Artificial preservatives and their harmful effects: Looking toward nature for safer alternatives. Int J Pharm Sci and Res. 4:2496-2501.

2. Angelino D, Dosz E, Sun J, Hoeflinger JL, Van Tassell ML, Chen P, Harnly JM, Miller MJ, Jefferey EH. Myrosinase-dependent and –independent formation and control of isothiocyanate products of glucosinolate hydrolysis. Front Plant Sci. 6: 831.

3. Badi HN, Yazdani D, Ali SM, Nazari F. 2004. Effects of spacing and harvesting time on herbage yield and quality/quantity of oil in thyme, *Thymus vulgaris L.* Ind Crops Prod. 19:231-236.

4. Barchiesi F, Colombo AL, McGough DA, Rinaldi MG. 1994. Comparative study of broth macrodilution and microdilution techniques for in vitro antifungal susceptibility testing of yeasts by using the National Committee for Clinical Laboratory Standards proposed standard. J Clin Microbiol. 32:2494-2500.

5. Bauermeister LJ, Bowers JWJ, Townsend JC, McKee SR. 2008. The microbial and quality properties of poultry carcasses treated with peracetic acid as an antimicrobial treatment. Poult Sci. 87:2390-2398.

6. Bayramoglu B, Sahin S, Sumnu G. 2008. Solvent-free microwave extraction of essential oil from oregano. J Food Eng. 88:535-540.

7. Burt SA, Reinders RD. 2003. Antibacterial activity of selected plant essential oils against *Escherichia coli* O157:H7. Lett Appl Microbiol. 36: 162-167.

8. Busatta C, Vidal RS, Popiolski AS, Mossi Aj, Dariva C, Rodrigues MRA, Corazza FC, Corazza ML, Oliveira JV, Cansian RL. 2008. Application of *Origanum majorana* L. essential oil as an antimicrobial agent in sausage. Food Microbiol. 25:207-211.

9. Burt, S. 2004. Essential oils: their antibacterial properties and potential applications in food—a review. Int J Food Microbiol. 94: 223-253.

10. Cabral Marques HM. 2010. A review on cyclodextrins of essential oils and volatiles. Flavour Fragrance J. 25: 313-326.

11. Carocho M, Barreiro MF, Morales P, Ferreira ICFR. 2014. Adding molecules to food, pros and cons: A review on synthetic and natural food additives. Compr Rev Food Sci Food Saf. 13: 377-399.

12. Cava R, Nowak E, Taboada A, Marin-Iniesta F. 2007. Antimicrobial activity of clove and cinnamon essential oils against Listeria monocytogenes in pasteurized milk. J Food Prot. 70: 2757-2763.

13. CDC. 2017[a]. FoodNet data and reports. Available from: https://www.cdc.gov/foodnet/reports/index.html. Accessed 1 June 2017.

14. CDC. 2017[b]. Incidence and trends of infections with pathogens transmitted commonly through food and the effect of increasing use of culture-independent diagnostic tests on surveillance — foodborne diseases active surveillance network, 10 U.S. sites, 2013–2016. Available from: https://www.cdc.gov/mmwr/volumes/66/wr/mm6615a1.htm?s_cid=mm6615a1_w . Accessed 2 May 2017.

15. CDC. 2016. *Salmonella*: Questions and Answers. Available from: https://www.cdc.gov/salmonella/general/index.html. Accessed 17 January 2017.

16. CDC. 2015. *Salmonella*. Available from: https://www.cdc.gov/salmonella/general/technical.html. Accessed 17 January 2017.

17. CDC. 2015. Multistate outbreak of drug-resistant *Salmonella* Enteritidis infections linked to raw, frozen, stuffed chicken entrees produced by Barber Foods (Final Update). Available from: http://www.cdc.gov/salmonella/frozen-chicken-entrees-07-15/index.html. Accessed 17 January 2017.

18. CDC. 2014. Multistate outbreak of multidrug-resistant *Salmonella* Heidelberg infections linked to foster farms brand chicken (Final Update). Available from: http://www.cdc.gov/salmonella/heidelberg-10-13/index.html. Accessed 17 January 2017.

19. CDC. 2013. Multistate outbreak of *Salmonella* Typhimurium infections linked to ground beef (Final Update). Available from: http://www.cdc.gov/salmonella/typhimurium-01-13/index.html. Accessed 17 January 2017.

20. CDC. 2012. Multistate outbreak of *Salmonella* Typhimurium and *Salmonella* Newport infections linked to cantaloupe (Final Update). Available from: http://www.cdc.gov/salmonella/typhimurium-cantaloupe-08-12/index.html. Accessed 17 January 2017.

21. CDC. 2012. Multistate outbreak of *Salmonella* Enteritidis infections linked to ground beef (Final Update). Available from: http://www.cdc.gov/salmonella/enteritidis-07-12/index.html. Accessed 17 January 2017.

22. Chouliara E, Karatapanis A, Savvaidis IN, Kontominas MG. 2007. Combined effect of oregano essential oil and modified atmosphere packaging on shelf-life extension of fresh chicken breast meat, stored at 4 °C. Food Microbiol. 24:607-617.

23. David, JRD, Ekanayake A, Singh I, Farina B, Meyer M. 2013. Effect of white mustard essential oil on inoculated *Salmonella* sp. in a sauce with particulates. J Food Prot. 76: 580-587.

24. Delaquis PJ, Mazza G. 1995. Antimicrobial properties of isothiocyanates in food preservation. Food Technol. 49:73-74, 79,81,83-84.

25. Delaquis PJ, Mazza G. 1995. Antimicrobial properties of isothiocyanates in food preservation. Food Technol. 49: 73-79.

26. Diaz-Maroto MC, Perez-Coello MS, Cabezudo MD. 2002. Supercritical carbon dioxide extraction of volatiles from spices-Comparison with simultaneous distillation-extraction. J Chromatogr A. 947:23-29.

27. Diaz-Maroto MC, Hidalgo IJD-M, Sanchez-Palomo E, Perez-Coello MS. 2005. Volatile components and key odorants of fennel (*Foeniculum vulgare Mill.*) and thyme (*Thymus vulgaris L.*) oil extracts obtained by simultaneous distillation-extraction and supercritical fluid extraction. J Agri Food Chem. 53:5385-5389.

28. Donsi, F, Annunziata M, Sessa M, Ferrari G. 2011. Nano encapsulation of essential oils to enhance their antimicrobial activity in food. Food Sci and Technol. 44: 1908-1914.

29. Dorman HJD, Deans SG. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. J Applied Microbiol. 88:308-316.

30. Ekanayake A, Zoutendam PH, Strife RJ, Fu X, Jayatilake GS. 2012. Development of white mustard (Sinapis alba L.) essential oil, a food preservative. Food Chem. 133: 767-774.

31. Emiroglu, ZK, Yemis GP, Coskun BK, Candogan K. 2010. Antimicrobial activity of soy edible films incorporated with thyme and oregano essential oils on fresh ground beef patties. Meat Sci. 86:283-288.

32. FDA. 2014. Inventory of effective food contact substance (FCS) notifications. Available from: https://www.accessdata.fda.gov/scripts/fdcc/?set=FCN&id=1419. Accessed 5 June 2017.

33. FDA. 2016[b]. CFR-Code of Federal Regulations title 21. Available from: https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?CFRPart=179&showFR=1.

34. FDA. 2016[a]. Food Irradiation: What you need to know? Available from: https://www.fda.gov/Food/ResourcesForYou/Consumers/ucm261680.htm.

35. FDA. 2017. CFR-Code of Federal Regulations title 21. Available from: https://www.ecfr.gov/cgi-bin/text-

idx?SID=e956d645a8b4e6b3e34e4e5d1b690209&mc=true&node=pt21.3.182&rg n=div5#se21.3.182_11. Accessed 5 June 2017.

36. Falguera V, Quintiro JP, Jimenez A, Munoz JA, Ibarz A. 2011. Edible films and coatings: Structures, active functions and trends in their use. Trends Food Sci Technol. 22:292-303.

37. Friedman M, Henika PR, Levin CE, Mandrell RE. 2004. Antibacterial activities of plant essential oils and their components against *E. coli* 0157:H7 and *Salmonella enterica* in apple juice. J Agric Food Chem. 52: 6042-6048.

38. Gill AO, Holley RA. 2006. Disruption of *Escherichia coli*, *Listeria monocytogenes* and *Lactobacillus sakei* cellular membranes by plant oil aromatics. Int J Food Microbiol. 108:1-9.

39. Golmakani MT, Rezaei K. 2008. Comparison of microwave-assisted hydrodistillation with the traditional hydrodistillation method in the extraction of essential oils from *Thymus vulgaris L*. Food Chem. 109: 925-930.

40. Gutierrez, J, Barry-Ryan C, Bourke P. 2008. The antimicrobial efficacy of plant essential oil combinations and interactions with food ingredients. Int J Food Microbiol. 124: 91-97.

41. Helander IM, Alakomi HL, Latva-Kala K, Mattila-Sandholm T, Pol I, Smid EJ, Gorris LGM, von Wright A. 1998. Characterization of the action of selected essential oil components on gram-negative bacteria. J Agric Food Chem. 46:3590-3595.

42. Hill LE, Gomes C, Taylor TM. Characterization of beta-cyclodextrin inclusion complexes containing essential oils (trans-cinnamaldehyde, eugenol, cinnamon bark, and clove bud extracts) for antimicrobial delivery applications. Food Sci Technol. 51:86-93.

43. Hyldgaard M, Mygind T, Meyer RL. 2012. Essential oils in food preservation: Mode of action, synergies and interactions with food matrix components. Front Microbiol. 3:1-26.

44. Jackson BR, Griffin PM, Cole D, Walsh KA, Chai SJ. 2013. Outbreak-associated *Salmonella enterica* serotypes and food commodities, United States, 1998–2008. Emerg Infect Dis. *19*: 1239-44.

45. Juven BJ, Kanner J, Schved F, Weisslowicz H. 1994. Factors that interact with the antibacterial action of thyme essential oil and its active constituents. J Applied Microbiol. 76: 626-631.

46. Kitis, M. 2004. Disinfection of washwater with peracetic acid: a review. Eviron Int. 30:47-55.

47. Lambert RJW, Skandamis PN, Coote PJ, Nychas GJE. 2001. A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. J Appl Microbiol. 91: 453-462.

48. Liang H, Yuan Q, Vriesekoop F, Lv F. 2012. Effects of cyclodextrins on the antimicrobial activity of plant-derived essential oil compounds. Food Chem. 135:1020-1027.

49. Lin CM, Preston III JF, Wei CI. 2000. Antibacterial mechanism of allyl isothiocyanate. J Food Prot. 63:727-734.

50. Luciano FB, Holley RA. 2009. Enzymatic inhibition by allyl isothiocyanate and factors affecting its antimicrobial action against *Escherichia coli* O157:H7. Int J Food Microbiol. 131:240-245.

51. McGimpsey JA, Douglas MH, Van Klink JW, Beauregard DA, Perry NB. 1994. Seasonal variation in essential oil yield and composition from naturalized *Thymus vulgais L.* in New Zealand. Flavour Fragrance J. 9: 347–352

52. Madigan MT, Martinko JM, Stahl DA, Clark DP. 2012. Food preservation and foodborne microbial diseases. Brock biology of microorganisms. Thirteenth Edition. Boston: Benjamin Cummings. p 1036-1037.

53. Mead PS, Slutsker L, Dietz V, McCaig LF, Bresee JS, Shapiro C, Griffin PM, Tauxe RV. 1999. Food-related illness and death in the United States. Emerg Infect Dis. 5: 607-625.

54. Montville TJ, Matthews KR, Kniel KE. 2005. *Salmonella* species. Food microbiology an introduction. Third Edition. Washington D.C: ASM Press. p 208-217.

55. National Center for Biotechnology Information. PubChem Compound Database. Available from: https://pubchem.ncbi.nlm.nih.gov/compound/160611. Accessed June 27, 2017.

56. Nazer AI, Kobilinksy A, Tholozan JL, Dubois-Brissonnet F. 2005. Combinations of food antimicrobials at low levels to inhibit the growth of *Salmonella* sv. Typhimurium: a synergistic effect? Food Microbiol. 22:391-398.

57. Negi PS. 2012. Plant extracts for the control of bacterial growth: Efficacy, stability and safety issues for food application. Int J of Food Microbiol. 156: 7-17.

58. Nielsen PV, Rios R. Inhibition of fungal growth on bread by volatile components from spices and herbs, and the possible application in active packaging, with special emphasis on mustard essential oil. Int J Food Microbiol. 60:219-229.

59. Nychas GJE, Tassou CC. 1996. Growth/survival of *Salmonella* enteritidis on fresh poultry and fish stored under vacuum or modified atmosphere. Lett Appl Microbiol. 23: 115-119.

60. Olaimat AN, Holley RA. 2013. Effects of changes in pH and temperature on the inhibition of *Salmonella* and *Listeria monocytogenes* by Allyl isothiocyanate. Food Control. 34:414-419.

61. Olasupo NA, Fitzgerald DJ, Gasson MJ, Narbad A. 2003. Activity of natural antimicrobial compounds against *Escherichia coli* and *Salmonella enterica* serovars Typhimurium. Lett Appl Microbiol. 37:448-451.

62. Olsen SJ, Bishop R, Brenner FW, Roels TH, Bean N, Tauxe RV, Slutsker L. The Changing epidemiology of *Salmonella*: Trends in serotypes isolated from humans in the United States, 1987-1997. J Infect Dis. 183: 753-761.

63. Over KF, Hettiarachchy N, Johnson MG, Davis B. 2009. Effect of organic acids and plant extracts on Escherichia coli O157:H7, *Listeria monocytogenes*, and

Salmonella Typhimurium in broth culture model and chicken meat systems. J Food Sci. 74:M515-M521.

64. Pintar K, Cook A, Pollari F, Ravel A, Lee S, Odumeru JA. 2007. Quantitative effect of refrigerated storage time on the enumeration of *Campylobacter*, *Listeria*, and *Salmonella* on artificially inoculated raw chicken meat. J Food Prot. 70: 739-743.

65. Russo M, Galletti GC, Bocchini P, Carnacini A. 1998. Essential oil chemical composition of wild populations of Italian oregano spice (*Origanum vulgare ssp. hirtum* (Link) Ietswaart): A preliminary evaluation of their use in chemotaxonomy by cluster analysis: 1. Inflorescences. J Agric Food Chem. 46: 3741–3746.

66. Silva, F, Domingues FC, Nerin C. 2016. Trends in microbial control techniques for poultry products. Crit Rev of Food Sci and Nutr.

67. Senatore F. 1996. Influence of harvesting time on yield and composition of the essential oil of a thyme (*Thymus pulegioides L.*) growing wild in Campania (Southern Italy). J Agric Food Chem. 44: 1327–1332.

68. Seydim AC, Sarikus G. 2006. Antimicrobial activity of whey protein based edible films incorporated with oregano, rosemary and garlic essential oils. Food Res Int. 39: 639-644.

69. Skandamis PN, Nychas GJE. 2000. Development and evaluation of a model predicting the survival of *Escherichia coli* O157:H7 NCTC 12900 in homemade eggplant salad at various temperatures, pHs and oregano essential oil concentrations. Appl Environ Microbiol. 66:1646-1653.

70. Solomakos N, Govaris A, Koidis P, Botsoglou N. 2008. The antimicrobial effect of thyme essential oil, nisin, and their combination against *Listeria monocytogenes* in minced beef during refrigerated storage. Food Microbiol. 25: 120-127.

71. Tassou CC, Drosinos EH, Nychas GJE. 1995. Effects of essential oil from mint (*Mentha piperita*) on *Salmonella* Enteritidis and *Listeria monocytogenes* in model food systems at 4°C and 10°C. J Appl Bacteriol. 78:593-600.

72. Techathuvanan C, Reyes F, David JRD, Davidson MP. 2014. Efficacy of commercial natural antimicrobials alone and in combinations against pathogenic and spoilage microorganisms. J Food Prot. 77:269-275.

73. Tsao R, Yu Q, Friesen I, Potter J, Chiba M. 2000. Factors affecting the dissolution and degradation of oriental mustard derived sinigrin and allyl isothiocyanate in aqueous media. J Agri Food Chem. 48:1898-1902.

74. USDA Food Safety And Inspection Service Microbiology Division. 2017. Isolation and identification of *Salmonella* from meat, poultry, pasteurized egg, and siluriformes (fish) products and carcass and environmental sponges *In* Microbiology Laboratory Guidebook 4.09. Available at: https://www.fsis.usda.gov/wps/portal/fsis/topics/science/laboratories-and-procedures/guidebooks-and-methods/microbiology-laboratory-guidebook/microbiology-laboratory-guidebook.

75. Weiss J, Gaysinksy S, Davidson M, McClements J. 2009. Nanostructured encapsulation systems: food antimicrobials. In Barbosa-Canovas GV, Mortimer

A, LIneback D, Siess W, Buckle K, editors. IUFoST World Congress book: Global issues in food science and technology. Amsterdam: Elsevier Inc. p. 425-479.

76. Zivanovic S, Chi S, Draughon AF. 2005. Antimicrobial activity of chitosan films enriched with essential oils. J Food Sci. 70:M45-M51.