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# Antibacterial activity of essential oil encapsulated sodium iota-carrageenan fibers

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# ANTIBACTERIAL ACTIVITY OF ESSENTIAL OIL ENCAPSULATED SODIUM IOTA-CARRAGEENAN FIBERS

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of

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by

Carlos D. Carter

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For my family.

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# ABSTRACT

Carter, Carlos, Devon M.S., Purdue University, December 2016. Antibacterial Activity of Essential Oil Encapsulated Sodium Iota-Carrageenan Fibers. Major Professors: Dr. Arun K. Bhunia & Dr. Srinivas Janaswamy

Spoilage microorganisms cause food waste and loss of quality. While the foodborne pathogen outbreaks lead to thousands of hospitalizations and deaths. Essential oils (EOs), plant extracts, possess the required antimicrobial activities and thus their usage stands out as a feasible approach for controlling the undesirable bacterial growth in food systems. However, EOs are highly volatile and lose their activity upon exposure to environmental conditions. In this regard, their encapsulation in Generally Recognized As Safe (GRAS) matrices such as food grade polysaccharides especially iota-carrageenan could be one of the viable alternatives. Iota-carrageenan, sulfated polysaccharide from marine algae, is being used in food, pharmaceutical and medical application as a gelling and thickening agent. Ordered networks composed of water pockets of the dimensions of EOs could be created by stretching the oriented fibers of iota-carrageenan. These water pockets readily encapsulate the EOs, protect from external stresses, e.g. heat, light, moisture, and release in a controlled manner. Herein, two EOs, carvacrol and eugenol mixed in ethanol and Tween 80 were encapsulated in sodium salt form of iota-carrageenan fibers. The antimicrobial activity was tested

against *Listeria innocua* F4248, *Listeria monocytogenes* F4244, *Salmonella* Enteritidis ENT 1344, *Salmonella enterica* serovar Heidelberg 513, *Escherichia coli* O157: H7 ATCC 43295, and *Staphylococcus aureus* ATCC 25923 using the disc diffusion and macro broth dilution assay. Results reveal that the complexes dissolve in the deionized water and EO release in about 40 mins. The complexing, indeed, have inhibitory effect on the growth of the microorganisms, especially in significantly reducing the proliferation of *L. monocytogenes* and *L. innocua*. Overall, the intrinsic functionality of essential oils could be preserved by encapsulating them in the ordered polysaccharide matrices for inhibiting the growth of spoilage microorganisms in food systems.

# CHAPTER 1. REVIEW OF ESSENTIAL OILS: COMPOSITION, CURRENT APPLICATIONS, AND POTENTIAL DELIVERY SYSTEMS FOR FOOD

## 1.1 Introduction

Foodborne illnesses are major issues in the world today. In year 2013, the Center for Disease Control (CDC) reported 19,162 laboratory-confirmed cases of infection, 4,276 hospitalizations and 88 deaths [FoodNet, 2016]. The CDC also estimates every 1 in 6 Americans are infected with a foodborne illness. The top contributing pathogens are *Salmonella*, *Listeria monocytogenes*, *Campylobacter* spp., *Toxoplasma gondii*, and the Norovirus. Food is generally contaminated during processing and handling - person with poor hygiene - cross contamination and poor sanitation in facilities. Bacterial persistence and resistance has also been a growing challenge in the agricultural and food industry. Fortunately, the government, food industry, and universities have made great strides in reducing foodborne illness outbreaks through research.

In 2011, the Food Safety Modernization Act (FSMA) was signed and it shifted the focus from responding to contamination to preventing it. The FSMA further strengthens the food system by allowing the FDA to mandate new legislation on contamination prevention, inspection & compliance, response, imported products, and enhanced partnerships with state and local agencies (U.S. Food and Drug Admin).

According to the CDC, in 2012, laboratory-confirmed infections for *Listeria*, *Shigella*, Shiga toxin-producing *E. coli* O157:H7, and *Yersinia* decreased by about 6, 13, 10, and 6%, respectively. However, confirmed infections for *Campylobacter*, *Salmonella*, and *Virbrio* increased by about 14, 3, and 43%, respectively. Microbial contamination and spoilage continue to be issues to be addressed in the agricultural and food sectors around the world. Thus, there is further scope for improving the current traits so as to decrease the food borne illness outbreaks.

Many research groups have explored the option of incorporating the antimicrobial compounds (AMCs) into food matrix towards limiting the pathogenic growth. Among the several available AMCs, essential oils (EOs) have found an indispensable niche in the food safety and preservation. EOs are aromatic liquids obtained from plants. They can be obtained by expression, fermentation, or steam distillation (Burt, 2004). EOs have been recognized for their antimicrobial activity for centuries. They were primarily used for medicinal and pharmaceutical purposes. However, stability has been an issue in introducing EOs in food systems as they are highly volatile and can be easily oxidized by light. Additionally, high levels of fat and/or protein in food could shield bacteria from the action of EOs or interact with them and reduce the antimicrobial effect. Consequently, increased amounts of EOs have to be used to achieve the same antimicrobial activity effect. However, the organoleptic impact of pungent EOs should be considered, as they tend to alter the taste and smell or as well exceed the acceptable flavor thresholds. In this regard, various encapsulation methods have been employed to alleviate the challenges of EOs addition to food

systems. Rest of the section will focus on the functional components, current applications and delivery systems of EOs

## 1.2 History of Essential Oils (EOs)

The production of oils from distilled plant material dates all the way back to more than 2,000 years. Greek historian, Herodotus (484-425 B.C.), Roman historian Pliny (23-79) and Dioscorides mention oil of turpentine in their literature, but with limited information on how it was produced. During the Middle Ages, rose oil was regarded as an undesirable byproduct from producing distilled rose water. Odoriferous oils and ointments were traded in ancient Greece and Rome; however, they were not real EOs. The oils traded were produced by placing flowers, roots and other plant material into a fatty oil of high quality, exposing the glass bottles to the sun and then separating the oil from the solid constituents (Guenther, 1948). The first description of real EOs from distillation has generally been attributed to Catalan physician, Arnald de Villanova who may have also introduced distillation into European therapy. It should be noted the term "distilled" does not have the same meaning now as it did in ancient and medieval writings, and it is possible that modern distilled EOs might possess different contents than the ancient ones.

Significant development of oils began in the thirteenth century, when pharmacies started to prepare "remedy oils' and studied the properties and physiological effects (Surburg, 2006). The actual use of essential oils does not become common until and after the scientific discoveries made during the 21$^{st}$ century. Hieronymus Brunshwig's mentions oils of turpentine, juniper wood and rosemary in his

book “Liber De Arte” (Guenther, 1948). Loncier addresses the medicinal value of EOs and further expresses that distillation is rather a recent invention but not as an ancient invention (Guenther, 1948). The definition of distillation indeed changed over time and scientific advancement. German physician Valerius Cordus brought more knowledge on the nature and preparation of essential oils in the “De Artificiosis Extractionibus” (Guenther, 1948). Neapolitan, Giovanni Battista della Porta also wrote a very important publication on essential oils, ‘De Destillatione libri IX,”. Porta differentiates between fatty and distilled oils, their preparation, the ways of separating the volatile oils (Guenther, 1948).

The systematic study of EOs began towards the end of the 18$^{th}$ century. Lavoisier and Houton de la Billarddiè analyzed oil of turpentine and found that the ratio of carbon to hydrogen to be five to eight and the same ratio was also found in all hermiterpenes, terpenes, sequiterpenes and polyterpenes (Guenther, 1948). French Chemist Dumas also analyzed various stearoptenes (Guenther, 1948). Berthelot focused on the hydrocarbons in EOs (Guenther, 1948). Further advancement led to more discoveries and production of newly identified constituents. Some researchers refer to this period of advancement as the “Elizabethan Age” of EOs. The industrialization of EOs was indeed an important step in the history of natural fragrance materials.

The first EO produced on a large scale in the US was oil of turpentine. This was due to many southern states being covered with pine forest like North and South Carolina and Georgia as well as the national and global demand for EOs (Guenther, 1948). Additionally, oils from three American plants of sassafras, wormseed and

wintergreen were also produced in large scale. Oils of wintergreen and wormseed were highly used in North America. Williams Proctor, Jr. also known as “The Father of American Pharmacy” identified the main components of the oils of wintergreen (Guenther, 1948). The wintergreen oils gained medical, cosmetic and flavor applications. There is no evidence of large-scale production of wintergreen oil before the nineteenth century apart from the US (Guenther, 1948). James Thatcher noted in 1821 that oil from wormseed is one of the most efficacious vermifuge medicines ever employed (Guenther, 1948). Additionally, the oil of peppermint was also produced in bulk in North America. It is believed that the American distillation of peppermint oil on a commercial scale had its origin in New York during 1816 (Guenther, 1948). In 1850, single organic compounds were employed as fragrance materials. This resulted from the isolation of cinnamaldehyde from cinnamon oil (Dumas and Pèligot, 1834) and benzaldehyde from almond oil (Surburg and Panten, 2006). Certainly, the vast production of EOs took place during the twentieth century.

### 1.3 Current applications of EOs

EOs have been employed for various applications in food, pharmaceutical and perfumes. Single components of EOs have been isolated and used in flavoring or perfumery. Geraniol is isolated from cintronella oils and in small quantities to emphasize citrus notes. Menthol is isolated from cornmint oil and is used for its refreshing effect in cigarettes, toothpastes, chewing gum, snacks, and medicine (Surburg and Panten, 2006). EOs are also popular in the aromatherapy market holding over 2% of the total market. EOs and their components, either plant extracts or synthetically produced, have also

been used as food flavorings (Burt, 2004). Their antimicrobial properties and components have been adopted for various applications.

Many EOs are used as flavoring agents in food categories like meat, alcoholic beverages, soft drinks and savory foods. The pharmaceutical industry added EOs and their derivatives in cough medicines to mask undesirable odors and flavors. EOs have also been used in pharmaceutical preparations of ointments and lotions for dermatological disease and skin healing effects. EOs of elemi have been used as a diagnostic reagent in blood test. Interestingly, oils of calamintha have been used to lure wild cats (Surburg and Panten, 2006). In the veterinary field, EOs have been used as preventatives of ticks and fleas. Additionally, they have been employed as a prophylactic, in incipient paralysis, for rheumatism and arthritis and to get rid of lice. Domca, in Alhendin, Granada, Spain produces DMC Base, which comprises of 50% EOs like rosemary, sage and citrus. Bavaria Corp. in Apopka, FL produces Protecta One and Proteca Two which are blended herb extracts and are Generally Recognized As Safe (GRAS) as food additives in the US (Burt, 2004).

### 1.4 Composition of EOs

EOs are synthesized by all the plant organs (flowers, buds, seeds, leaves, twigs, bark, herbs, wood, fruits and roots) and are stored in secretory cells, cavities, canals, or glandular trichomes. The proportions of the components present vary greatly. Factors, like the plant type, geographic conditions, climate, harvest period and processing technique could have an effect on the chemical composition. In general, they have a complex composition and contain several hundred different components. The majority

includes terpenes, monoterpenes, and sequiterpenes. Additionally, allyl- and propenylphenols are also important components. Their antioxidant activity correlates well with the content of oxygenated phenolic monoterpenes (Bozin *et al.,* 2006). The individual contents play specific roles in the properties such as antimicrobial, antioxidant, and anti-inflammatory. Although numerous EOs have been chemically characterized, as of today, the composition-functionality relationship is not well understood yet.

### 1.4.1 Antibacterial Components

The antibacterial activity of many EOs or EO components has already been demonstrated by many research groups. There is some evidence that minor components play a vital role in the antimicrobial activity, producing a synergistic effect with major components. It has been reported that the phenolic components of essential oils are responsible for the antibacterial properties (Burt, 2004). In general, antimicrobial activity can be attributed to more than one component of an essential oil. For example, oil from *Thymus vulgaris* (Thyme) contains thymol, carvacrol, γ-Terpiene, and p-Cymeme, and all have been proven to be antibacterial. Origanum vulgare (oregano) also contains these same components in different amounts. Rosmarinus officinalis (rosemary) also contains four antimicrobial compounds, α-pinene, Bornyl acetate, Camphor, and 1,8-cineole (Burt 2004).

The EOs and EO components are lethal to bacteria at various concentrations. Baydar *et al*., 2004 showed oil from oregano and black thyme created larger zones of inhibition than wild oregano at various concentrations. These oils contained high

amounts of carvacrol. Oils from medicinal plants from the Democratic Republic of Congo were screened against several bacterial species. The most antibacterial EOs were those isolated from *Eucalyptus camadulensis* and *Eucalyptus terticoris* (12-30 mm zone of inhibition diameter) (Cimanga, 2010). Other EOs show exceptional antibacterial activity as well (≤ 15mm zone of inhibition diameter). *Eucalyptus propinqua*, *Eucalyptus urophylla* and *Ocimum gratissimum* oils weren't as effective as others in this study. They reported that no correlation was observed between the amount of major constituents such as 1,8-cineol, α-pinene, p-cymeme, cryptone, or thymol and the antibacterial activity (Cimanga, 2010).

Three EOs obtained from different species of Thymus plants growing wild in Sardinia was analyzed by GC/MS and their antimicrobial activity was determined against multiple strains of bacteria (Cosentino, 1999). The results showed the species had comparable antimicrobial activity to the commercial reference, *Thymus capitatus*. The major components of the oils were α-pinene, thymol, and carvacrol. Interestingly, the reference EO contained higher amounts of α-pinene (25.2%) than the wild EOs (0.8-1.9%) (Cosentino, 1999).

Eleven EOs (*Cananga odorta, Cupressus sempervirens, Curuma longa, Cymbopogon citratus, Eculyptus globulus, Pinus radiate, Piper crassinervium, Psidium quayava, Rosmarinus officinalis, Thymus citriodorus, Zinger officinale)* were screened to test for antioxidant, antiradical, and antimicrobial activity. The minimum inhibitory concentration of the oils was determined by the antimicrobial disc diffusion assay against 5-food spoilage yeast. Most EOs tested showed moderate antimicrobial activity

against the yeast. *Piper crassinervium*, which was never analyzed before, contained high amounts of limonene (26.6%), α- and β-pinene (10.0% and 15.2%, respectively). There weren't many differences observed in composition by *C. citratus, C. sempervirens, E. globulus, C. odorata*. The composition of other oils was unique (Sacchetti, 2005).

In a study, sodium nitrate was used to induce the inhibition of *Lactobacillus* growth by monolaurin and eugenol. Combinations of 100 to 250 ppm monolaurin with 500 and 100 ppm eugenol, and 0.2% were more effective at preventing detectable growth of 5 meat spoilage and 2 pathogenic organisms (Blaszyk, 1998). Microemulsions composed of eugenol, poly (vinyl alcohol), and Surfynol ® 465, were electrospun to create nanofiber carrier systems. The eugenol nanofibers were successful at suppressing the growth of *Salmonella enterica* serovar Typhimurium (2476 and 2576) and *Listeria monocytogenes* (Scott A and 101) (Kriegel, 2010).

EOs from lemon, sweet orange and bergamot and their components, linalool and citral had antibacterial effects as direct oil and vapor form against *Campylobacter jejuni*, *E. coli* O157, *L. monocytogenes*, *Bacillus cereus* and *S. aureus*. Only linalool, bergamot, and citral had MICs acceptable for food application (0.06-0.125% v/v, 1-0.125% v/v, and 0.03-0.06% v/v, respectively) (Fisher and Philips,2006).

### 1.4.2 Antioxidant Components

The antioxidant activity of EOs has been examined by many using various methods. Yang *et al.* (2010) found that *Lanvandula angustifolia* Mill oil from Australia was effective against lipid peroxidation (Yang, 2010.) The main contents of this oil are linalool and lynalyl acetate. Wei and Shibamoto (2010) evaluated the antioxidant

activity of oils from thyme, cloves, and basil using the conjugated diene assay. All oils had comparable antioxidant activity levels comparable to α-tocopherol, a common antioxidant. By conducting the β-carotene test, Joshi *et al.* (2010) showed that oils of *Dodecadenia grandiflora*, *Lindera pulcherrima*, and *Persea gamblei* were able to inhibit the oxidizing activity of the radical linoleic acid peroxide. Sesquiterpenoids were the major contents in this oil. Furanodienone and germacrene D were main components of Dodecadenia grandiflora, while furanodiene and curzerenone were the main components of *Lindera pulcherrima* oil. Oils from *Persea gamblei* were constituted by β-caryophyllene, γ-gurjunene and β-cubenee. Mighri *et al.* (2010) studied the oils of *Artemisa herbalba* also using the bleaching test. The results suggest four oil types β-thujone, α-thujone, thujones (α + β), and 1,8 cineole/ camphor/ thujones (α + β) that exhibited weak antioxidant activity that was attributed to the absence of nonphenolic compounds.

Using thiobarbituric acid reactive substances, Patil *et al,* (2010) showed oils from *Ageratum conyzoides* prevented lipid peroxidation better than the reference Butylated hydroxyanisole (BHA) (Patil, 2010). These oils were dominated by precocene I and precocene II. The antioxidant activity of five spice plants in the Mediterranean diet was also studied using the TBARS test (Viuda-Martos, 2010). All essential oils tested *(Thymus vulgaris*., [*Eugenia caryophyllus* (C. Spreng) Bell et Hare], *Origanum vulgare* L., *Salvia officinalis* L. and *Rosmarinus officinalis* L.) had antioxidant activity, but *Thymus vulgaris* was the most comparable to the reference chemical, butylated hydroxytoluene. The spices were predominated by Terpinen-4-ol, γ-terpinene, *cis*-sabinene hydrate, linalool

and *p*-cymeme. Oils from *Capparis spinosa* L. and *Crithmum maritimum* were evaluated by the TBARS method. They found that at the highest concentration, the oils showed lower ability to inhibit lipid peroxidation than butylated hydroxyanisole, but close to that of butylated hydroxytoluene. The differences among the antioxidant activity were not significant as compared to the chemical compositions. The major component in *Capparis spinose* oil was methyl isothicynate, while *Crithmum maritimum* oil was dominated by sabiene and limonene (Kulisic-Bilusic, 2010). On contrary, Miguel *et al.* (2010) showed oils from various aerial parts and seeds from *Foeniculum vulgare* Mill were pro-oxidant at higher concentrations (750 mg/ml for the areal parts and 1,000 mg/L for seeds). Aerial parts of the plant were dominated by *trans*-anethole and seeds by methyl chavicol.

The aldehyde/carboxylic acid assay is convenient for evaluating the effects of antioxidants against slow oxidation that occur over longer periods of time in foods (Miguel, 2010). Using this method, *Thymus vulgaris*, *Eugenia caryophyllus*, *Cinnamomum zeylanicum* Blume. *Ocimum basilicum* L and *Illicum verum* Hook f. prevented oxidation of hexanal to hexanoic acid after 40 days of storage. Interestingly, the results showed the low importance of phenolic compounds to prevent hexanal oxidation, as *Illicium verum* oil was primarily dominated by anethole (Wei and Shibamoto, 2010).

The formic acid measurement method is an automated test that measures the conductivity of low molecular weight fatty acids (formic acid) produced during oxidation of lipids (Miguel, 2010). The antioxidant activity of five spice plants found in the

Mediterranean diet was evaluated by the formic acid measurement. The results showed the oils of *Thymus vulgaris* and *Origanum vulagre* L. showed the best antioxidant activity, but less than the reference, BHT. Carvacrol was the main component of *Origanum vulagare* L. showed the greatest antioxidant activity (Viuda-Martos, 2010).

### 1.4.3 Anti-inflammatory Components

EOs are known to possess anti-inflammatory properties. Archidonis acid is released from cell membranes in inflammatory response. It is metabolized by Lipoxygenase pathways (Miguel, 2010). Essential oils from *Citrus aurantiuum* subsp. *bergamia,* Cinnamomun zeylanicum Blume, *Eucalyptus globus* Labill, Juniperus communis L. and Thymus vulgaris L., showed strong lipoxygenase inhibitory effects. These oils were dominated by limonene, linalyl acetate, β-*trans*-caryophyllene, 1, 8-cineole, *p-cymeme*, thymol and eugenol. The authors attributed the anti-inflammatory activity to 1, 8-cineole, β-trans-caryophyllene, and α-pinene. Chamazulene and α-bisabolol were major components of oils from the four plants of the *Helichrysum* species in South Africa in the inhibition of 5-lipoxygenase. Those same components were also present in chamomile essential oil. EOs of Alpinia murdocii Ridl., Alpinia scarbra, and Alpinia pahangeneis Ridl have also been shown to be good 5-lipoxygease inhibitors. Oils from the leaves were dominated by β-Pinene, α-pinene and sabiene, while the rhizome oils were dominated by γ-selinene, α-selinene and α-panasinsen (Kamatou, 2006).

Anti-inflammatory agents can inhibit the secretion of pro-inflammatory cytokines (Miguel, 2010). Interlukin-1β (IL-1β) and tumor necrosis factor-α (TNF- α) are cytokines that play major role in inflammatory response. Monocytes/ macrophages are

the major sources of TNF- α in inflammatory responses. IL-1β can be produced by a various cell lines like monocytes, macrophages, fibroblasts, and endothelial cells.

Researchers found that oil from *Melaleuca alternifolia* Cheel was able to suppress the production of TNF-α and IL-1β *in vitro*. The oil was predominated by terpinen-4-ol (Hart, 2006). EO of *Cheistocalx operculatus* inhibited lipopolysaccharide (LPS)-induced secretion of TNF- α and IL-1β (Dung, 2006). The same species reportedly also suppressed pro-inflammatory cytokine and stimulated the secretion of the anti-inflammatory IL-4 and IL-10. EO from *Taxandrua fragans* decreased the production of TNF-α and IL-6. It was mainly composed of 1,8-cineole and α-linalool (Hammer, 2008).

EO extracted from the leaves of *Cinnamomum osmophleom* had the ability to inhibit the production of IL-1β and IL-6 but not TNF- α. This was attributed to the content of 1,8-cineole, santoline, spathulenol and caryophyllene oxide. The anti-inflammatory activity of this species could also be due to cinnamaldehyde (Chao, 2005). Cinnamaldehyde isolated from leaves of *Cinnamomum osmophloeum* inhibited the secretion of IL-1β and TNF- α in multiple cell lines (Chao, 2005). Interestingly, oil from *Cordia verbenacea* reduced TNF- α levels, but not IL-1β (Passos, 2007). Other researchers showed that α-humelene isolated from the same species significantly reduced TNF- α and IL-1β levels in the tissue of the rat paw (Medeiros, 2007). Some single components of EOs could be more effective for specific functions (antimicrobial, antioxidant, anti-inflammatory).

Using an immunoassay, some researchers reported that EO from *Cryptomeria japonica* inhibited IL-1β, TNF- α, and IL-6 remarkably. This oil mainly contained kaurene,

elemol, γ-eudesmol and sabiene (Yoon, 2009). These same cytokines were also inhibited by oil from *Artemisia fukdo*, mainly dominated by α-Thujone, β-thujone, camphor and caryophyllene (Yoon, 2010). Main constituents of *Cymbopogon citratus* oil, geranial and neral, inhibited the secretion of IL-1β and IL-6 (Sforin, 2009). They later reported eugenol isolated from *Syzgium aromaticum* inhibited the release of $PGE_2$, TNF-α, and IL-1β (Rodrigues, 2009). Lin *et al.* reported, citral, the main component in oil from *Cinnamomum isularimontanum* Hayata, inhibits TNF-α. IL-1 and TNF-α levels were reduced in mice treated with oil from *Pterodon emarginatus*. The main components were germacrene D, β-elemene and caryophyllen. Combinations of thyme and oregano oils were capable of reducing levels of IL-1β and IL-6 (Dutra, 2009).

### 1.5 Encapsulation and Carrier Systems for EOs

As stated previously, incorporation of EOs and EO components has been a challenge for the food industry. High concentrations of EOs have to be used in food to retain their functional properties. EOs are very pungent and could change the organoleptic properties of foods. Additionally, food ingredients can protect the pathogenic and food spoilage microorganisms from the action of EOs (Gutierrez, 2008). Consequently, encapsulation and delivery systems for EOs has intrigued the interest of many researchers. Encapsulation, microemulsions, electrospraying, and electrospinning has been used to advance the application of EO and EO components in food. These methods could be useful in incorporating EOs in food to extend the shelf life and making food more functional.

### 1.5.1 Encapsulation of EOs

Oils isolated from oregano red thyme, and cassio have been encapsulated into zein nanospheres. Scanning Electron Microscopy (SEM) images indicated that the powders were made up of irregularly shaped particles, approximately (50 µm) containing closed-packed nanospheres (Parris, 2005). Oils extracted from eucalyptus and lemon peel were encapsulated as a mean to control their release. The $SiO_2$ were prepared by employing a sol-gel method to oil-in-water-in-oil multiple emulsions. The release profiles indicated depended on the chemical properties of each component (Sousa, 2014).

Essential oils distilled from *Artemisia afra*, *Eucalyptus globulus* and *Melaleuca alternifolia* were encapsulated into diasteroyl phosphatidycholine and phosphatidylethanolamine liposomes employing a reverse phase evaporation technique. The MIC assay results showed that *E. globulus* and *M. alternifolia* encapsulated liposomes were able to inhibit the microbial growth comparable to non-encapsulated oils. The addition of the chitosan coating did enhance the antimicrobial activity of the liposomes and had positive effect on the membrane stability (van Vuuren, 2010). Thyme essential oils were encapsulated into chitosan-benzoic acid nanogel (CS-BA) to enhance the antimicrobial activity against *Aspergillus flavus.* Under sealed conditions, the MIC of the CS-BA encapsulated essential oils was recorded at 300 mg/l while the nonencapsulated Thyme oil MIC 400 mg/l. Under non-sealed condition, the MIC of encapsulated thyme oils and non-encapsulated thyme oils were 500 mg/L and

1000 mg/l, respectively. Interestingly, *in vivo* test revealed significant anti-fungal properties of CS-BA encapsulated EOs at concentrations above 700 mg/l (Khalili, 2015).

Chitosan-Alginate nanocapsules were formulated to encapsulate turmeric and lemongrass oil. Results showed that 0.3 mg/mL alginate and 0.6 mg/mL chitosan produced minimum sized particles (<300 nm) with great stability. It was also reported, the nanocapsules were hemocompatible, suggesting potential for biomedical and pharmaceutical applications. Antiproliferative activity was also shown using the 3-(4,5-dimethy;thiazol-2-yl)-2,5,diphenyltetrzolium bromide assay. The nanocapsules were significantly more antiproliferative than the raw oils.

### 1.5.2 Essential Oils in Microemulsions

Formulation of microemulsions has been an avenue in applying EOs to foods. The Merriam Webster dictionary defines microemulsions as, “an emulsion in which the dispersed phase in the form of very small droplets usually produced and maintained with the aid of surfactants and having diameters of 50 to 500 angstroms. Sottmann and Stubenruach (2009) defines microemulsions as macroscopically isotropic mixtures of at least a hydrophilic, a hydrophobic and an amphiphilic component. Microemulsions could be vital in stabilizing EOs in food systems in liquid and solid phases.

Zhang *et al.* (2014) formulated microemulsions for potential washing solutions for organic fresh produce production. The oils from clove bud, cinnamon bark, and thyme were mixed with sucrose octanoate ester (SOE) and soy lecithin at various mass ratios before dilution with water to 40% (w/w.) The EOs were then mixed with the surfactant solution by hand shaking. The formation of microemulsions was favored at

lecithin: SOE mass ratios at 4% clove bud oil 2:8 and 3:7, 4% cinnamon bark oil and 3% thyme oil at 2:8 and 1:9, respectively. Microemulsions comprised of polysorbate 80 (Tween 80 ™ 80) as a surfactant, water, and propylene glycol as the polar phase have also been prepared. The oil phase contained EOs from cinnamon bark, eugenol, or thymol and Soybean oil at mass ratios of 1:0, 2:1, or 4:1. Results showed that SBO added to EOs expands the regimes of microemulsions and reduced the droplet dimensions that were stable over 90 days (Qiumin and Zhong, 2016).

In a study on the fatty liver and dyslipidemia in rats, clove essential oil (CO) and eugenol were formulated in water-based microemulsions. Results showed that CO microemulsions (COM) and eugenol microemulsions had particles sizes 8.0 nm and 8.9 nm, respectively. Excess dilution and incubation of these microemulsions in 1.2 N HCl, to mimic stomach juice, for 5 hours, lead to an establishment of a sub population with larger particle sizes of diameters less 100.0 nm. Rats who were administered daily doses of COM and EM, produced improvement in all biological evaluations. The controlled group exhibited dyslipidemia, high plasma tumor necrosis factor-α, and liver dysfunction. There was no correlation in the biochemical parameters when the experimental groups were given the different formulations (Al-Okbi, 2014).

### 1.5.3 Electrospraying Essential Oils

Electrospraying is a method of liquid atomization by means of electrical forces. In this methodology, a liquid flows out of a capillary nozzle, which is maintained at high electrical potential, and forced by an electrical field to be dispersed into fine droplets. It is currently being researched for food and biomedical applications. The size of

electrospray droplets could range from hundreds of micrometers to several tens of nanometers. Droplet generation and size could be controlled via the flow rate of the liquid and the voltage being applied to the capillary nozzle (Jawrek & Sobczykl, 2008). No heat is used during the electrospraying process. More recently, this technique is being used to encapsulate health promoting compounds including EOs.

Ghayempour and Mortazavi (2014) created antibacterial micro-nanocapsules by electrospraying oils from peppermint. In this study coaxial jet electrospray technique was developed to prepare the capsules. Encapsulation was achieved by preparing an emulsion of peppermint oil with polyoxyethylene sorbitan monolaurate emulsifier as the core material with 2% (w/v) sodium alginate as the wall material. SEM images showed that small spherical macro-nanocapsules were created. In various samples, 72% to 96.4% of the oil was successfully encapsulated and non-degraded. Using the shake flask method, the encapsulated macro-nano capsules reduced bacterial growth by 100%. Sunflower oil based material was electrosprayed on to complex surfaces as coatings. Selected model surfaces were nickel membranes with large rectangular pores (13 µm width), polyether sulfone (PES) membranes with small interconnected pores (0.2 µm) and dense cellulose membranes. The EO coating material penetrated the pores of PES and nickel membranes, filling them up and thereby significantly decreasing water vapor permeation flux. However, the materials accumulate on the cellulose membrane and the resulting reduction in water vapor permeation rate was much lower (Khan, 2012).

### 1.5.4 Electrospinning of Essential Oils

Electrospinning is very similar to electrospraying in that an electrical current is applied to capillary nozzle. A direct electrical current (10-30kV) is applied to the polymer solution creating a charged and continuous jet. The electrified jet is stretched due to the electrostatic repulsions between the surface charges and evaporation of the solvent. Fibrous mats are collected on a collector plate. Electrospraying creates random spherical macro or nano-capsules while electrospinning creates well-organized fibrous mats. Indeed, electrospinning is a simple and rapid technique. Thus fabricated fibers display small diameters (10-1000 nm) and high surface-area-to-volume ratio.

In a two-part study, antimicrobial nanofibers were fabricated by solubilizing eugenol (0.75-1.5 wt%) in surfactant micelles (Surfynol 465; 5-10% wt%) to form eugenol microemulsions (Kriegel, 2009). The microemulsions were then mixed with nonionic synthetic polymer poly (vinly alcohol) (PVA) to induce fiber formation. The mean diameter of the obtained fibers ranged from 57 - 126 nm. The surface conductivity and viscosity of the polymer solutions increased, while surface tension decreased. The mean diameter of the nanofibers decreased with increasing surfactant concentration and decreasing eugenol concentration. Subsequent, transmission electron microscopy showed that microemulsion droplets were homogenously dispersed throughout the nanofibers (Kriegel, 2009). Later, the antimicrobial activity and release characteristics of the electrospun eugenol nanofibers were tested. A burst release of the dispersed eugenol was noticed and the release rate was dependent on the eugenol and surfactant concentration. The antimicrobial activity of nanofibers was

evaluated against *Salmonella* Typhimurium (2476 and 2576) and *Listeria monocytogenes* (Scott A and 101). The presence of nanofibers in bacterial suspensions suppressed the test pathogens and in some cases decreased initial cell numbers (Kriegel, 2010).

Rieger and Schiffman (2014) electrospun essential oil component, cinnamaldehyde into chitosan (CS) /poly(ethylene oxide) (PEO). CA (0.5 and 5%.0) was incorporated into CS-PEO into mats with diameters of approximately 50 nm. Release studies showed 5.0 CA fiber mats released higher amounts of CA-liquid (545% more) and CA-vapor (279% more) than the 0.5% fiber mats. The quick release of CA enabled high inhibition rates of *Escherichia coli* and *Pseudomonas aeruginosa*. Reiger *et al.* (2016) later successfully electrospun CA and hydrocinnamic alcohol (H-CIN) in CS-PEO. Viscosity stress sweeps determined how the oils affected solution viscosity and chain entanglement concentration. The maximum polymer: oil mass ratio electrospun was 1:3 and 1:6 for CS/PEO; CA and H-CIN, respectively; a higher chitosan degree of acetylation increased the incorporation of H-CIN.

Vanillin/ cyclodextrin inclusion complex nanofibers (vanillin/CD-IC NFs) were fabricated from the modified CD types (HPβCD, HPγCD and MβCD) in three different solvent systems (water, DMFand DMAc). The polymer-free vanillin/CD-IC NFs allowed a much higher loading of vanillin (~12% w/w) compared to polymeric nanofibers (~5%W/W). Maximum preservation of vanillin was observed for vanillin/MβCD-IC NFs, approximately ~85% w/w, while vanillin/HPβCD-IC NS and vanillin/HPγCD-IN NFs preserved ~75% vanillin (Celebioglu, 2016).

## 1.6 Conclusion

Natural preservatives are in high demand for food. Today's consumers are more conscious about what they consume and prefer things that come from the natural sources. Food spoilage and foodborne illness is still a global issue. Pathogenic microorganisms cause thousands of hospitalizations and deaths annually, food loss, and huge economic losses. Essential oils and EO components possess antimicrobial, antioxidant, and anti-inflammatory properties and thus they stand out as viable option for addressing the pathogenic issues. However, incorporation of EOs is a challenge as they are unstable, volatile, pungent and not water soluble. High amounts of EOs in foods could change the sensory attributes. Several methodologies such as encapsulation, microemulsions, eletrospraying, and electrospinning are being pursued to apply EOs to food. Though there has been tremendous success there are still some critical issues to be addressed and the future is set to formulate more stable and hydrophilic delivery systems that are human compatible, wide spread, inexpensive as well as simple to handle.

# CHAPTER 2. INTORDCUTION & METHODS

## 2.1 Introduction

Carrageenans are hydrocolloids consisting mainly of D-galactose and anhydro-galatose units. They are used as gelling, thickening and stabilizing agents in food products. They are also used in medicine, pharmaceutical formulations, and cosmetics (Necas, 2013). They are extracted from the seaweed for example, from the coast of North America and Europe. They occur within the cell wall of red seaweeds and are usually extracted from species of Chondrus, Eucheuma, Polyides, Irideae, and Hypnea. Since 1962, carrageenans have been suggested to be suitable for use in food by the Food Additives and Contaminants Committee (Fd. Cosmet. Toxical, 1971). The FAO/ WHO first allocated an acceptable daily intake (ADI) of 0 – 50 mg/kg. Data from the expert Committee showed that carrageenans are not absorbed when ingested by animals nor did they cause an increase in mortality (Fd. Cosmet. Toxical, 1971). Thus, carrageenan is classified as safe and used for commercial applications.

There are carbohydrate residues (e.g., xylose, glucose) and substituents (e.g., methyl ethers and pyruvate groups) present in carrageenan. Carrageenans are classified into to various types such as λ, κ, ι, ε, μ and as of today, there are 15 known varieties

Higher levels of ester sulfate mean lower solubility temperature and lower gel strength (Necas, 2013).

The most common types of carrageenans used in the food industry are kappa-, lambda-, and iota-carrageenan. They dissolve in water to form highly viscous solutions. Potassium, calcium, and sodium ions together produce high gel strengths. Gels made with kappa(κ)-carrageenan are the strongest of all carrageenan gels but they tend to synerese. Iota(ι)- carrageenan yields gelation best with calcium ions. The resulting gel is soft, resilient and has a good freeze-thaw stability. It does not synerese, presumably fewer junction zones are created compared to κ-carrageenan (Damodaran, 2008). The λ-carrageenan is also water-soluble but does not gel.

It has been shown that carrageenans can be encapsulated with health promoting molecules. Polysaccharides like carrageenan cannot grow as large crystals, but can be stretched into well-oriented fibers. When carrageenans are in fiber form they take on a helical structure. Interestingly, carrageenan fibers held at a relative humidity (RH) of 66-75% creates a network stabilized by a series of ordered water molecules and amorphous water pockets. In these water pockets are where health promoting substances can be deposited. These studies have also shown that carrageenan fibers can be paired with balancing cations like sodium, potassium, and calcium (Janaswamy and Youngren, 2012). When molecules enter the water pockets of the carrageenan lattice it changes the normal packaging arrangement of the carrageenan. X-Ray Fiber Diffraction has been used to examine the atomic structural variations among the various forms of carrageenans, especially in the fiber forms. Carrageenan- cation combinations that have

high gel strength are likely to be capable of being grown into fiber form (Janawamy and Youngren, 2012). Weak gels tend to break before being stretched into fibers.

In this study, we hypothesize that fibers can be fabricated using sodium-ι-carrageenan solutions and encapsulate with EO components, eugenol and carvacrol. The EO components will be loaded into the water pockets of the fibers and the fibers will adopt the antibacterial functionality of eugenol and carvacrol. We suggest the antimicrobial compounds can quickly release from fibers and inhibit the growth of pathogenic bacteria. The objectives of this study are to demonstrate that well-oriented carrageenan fibers encapsulated with essential oils can inhibit bacterial growth and determine the antibacterial activity against Gram-positive and Gram-negative bacterial strains.

## 2.2 Materials and Methods

### 2.2.1 Chemicals

All solutions were prepared with deionized water. Research grade ι-carrageenan sample (RE-PR-4018) was provided by FMC Corporation, USA. Sodium Chloride (NaCl) (X190-1Kg, Lot #; 1502C144), and eugenol (A14332, Lot #: 10172314) were purchased from VWR International (Batavia, IL) and Alfa Aesar, respectively. Carvacrol (Lot #: MKBP5684V) was purchased from Sigma-Aldrich (Milwaukee, WI). Agar, Bacteriological (Lot: 107530A) and Brain Heart Infusion (BHI) Broth (Lot #: 106517A) were purchased from Neogen Corporation (Lansing, Michigan). Bacto ™ Tryptic Soy Broth (TSB) (Lot #: 5215822) was purchased from Fischer Scientific (Hanover Park, IL). Tween 80, ethanol, and acetone were used to prepare encapsulation solutions.

Bacterial Test Cultures

To test the antibacterial activity of the fibers, *Salmonella enterica* serovar Heidelberg 513, *Salmonella* Enteritidis ENT 1344, *Escherichia coli O*157: H7 ATCC 43295, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* F4244*, and Listeria innocua* F4248 were used. The cultures were obtained from the Bhunia Lab culture collection, Department of Food Science, Purdue University.

Table 1. Bacterial Cultures tested in the study

| # | *Bacterial Strain* |
|---|---|
| *1* | *Salmonella enterica* serovar Heidelberg 513 |
| *2* | *Salmonella* Enteritidis ENT 1344 |
| *3* | *Escherichia coli* O157: H7 ATCC 43295 |
| *4* | *Staphylococcus aureus* ATCC 25923 |
| *5* | *Listeria monocytogenes* F4244 |
| *6* | *Listeria innocua* F4248 |

2.2.2 Preparation of Sodium Iota-Carrageenan (IC) Fibers

The experimental approach to engineer IC fibers and encapsulate EO components in the organized network was derived from Polowsky and Janaswamy, (2015). This, in part, requires well-organized fiber formation, orientation and crystallinity. Briefly, 100 mg of IC and 36 mg of NaCl were dissolved in 10 mL of deionized water. The homogeneous solution was heated at 90 °C for 45 minutes with intermediate vortexing then cooled to ambient temperature to obtain a viscous gel. In

actual fiber preparation, a 20 µl droplet of the IC gel was placed in between two glass rods in a fiber puller at 66% relative humidity (RH). Once the droplet dried partially (after around 3 – 4 hours), it was stretched to approximately 2 – 3 mm in length at regular intervals, and was allowed to dry further for 12 hours in the fiber puller. Then the fibers were cut loose from the glass rods and stored in a desiccator at 66% RH for further experimentation.

### 2.2.4 Preparation of Antibacterial Fibers

Several encapsulation solutions were used in the study to determine the most effective against the selected test organisms. Table 2 displays the various solutions and concentrations. All solutions were mixed until a homogenous mixture was obtained. The solutions were stored at ambient temperatures. Stretched fibers were immersed in the selected solution for 7 days at an ambient temperature. The complex fibers were then removed from the solution, dried, and equilibrated at 66% RH for further analysis.

Table 2. *Types of solutions and concentrations examined in this study*

| # | *Solution and concentration combination* |
|---|---|
| *1* | 3% water, 47% Eugenol, 50% Ethanol |
| *2* | 3% water, 47% Carvacrol, 50% Ethanol |
| *3* | 25% Carvacrol, 25% Ethanol, 50% Tween 80 |
| *4* | 50% Carvacrol, 50% Tween 80 |
| *5* | 30% Carvacrol, 70% Tween 80 |

2.2.5 Eugenol and carvacrol release from the complexes and encapsulated amount

Concentration testing was performed using a Beckman Coulter DU 730 UV/Vis spectrophotometer with disposable UV cuvettes. Initially, wavelength scan was performed in the range 200 to 600 nm for determining the optimum wavelength of absorbance (276 nm) for eugenol and carvacrol (270 nm). Subsequently, calibration curve was generated by dissolving known amounts of eugenol and carvacrol in isopropyl alcohol. Distilled deionized water was used as solvent for estimating the eugenol or carvacrol content in the complex. The fibers weighing 1.0 mg were placed at the bottom of the cuvette containing 600 µL of distilled deionized water. The spectrometer was zeroed before the measurements with cuvette containing only water. The absorbance of the sample was taken every 2 min for 40 mins. Eugenol and carvacrol amounts in the complexes were estimated using the calibration curve with the known absorbance. Results are presented with means of duplicate experiments.

### 2.2.6 Antibacterial Activity Assay

Disc diffusion assay was the first test chosen to examine the antibacterial activity of the fibers. *Listeria innocua* F4248 and Brain Heart Infusion Broth/Agar were chosen as the initial test microorganism and media, respectively. *L. innocua* was grown for approximately 18 h. Soft BHI agar (0.75% agar, Bacteriological) was microwaved until melting and allowed to cool slightly. *L. innocua* was inoculated at a concentration 1 x $10^6$ cfu/ml into 5 ml of soft agar. The inoculated soft agar was then poured onto the surface of a BHI agar petri dish and allowed to solidify. The control experimental groups were placed in the center of soft agar plates. The plates were incubated at 37$^o$C for 24 h. After several trials, the zone of inhibition assay was shown to not be a viable method to display the antibacterial activity of carrageen-based fibers encapsulated with eugenol or carvacrol. Carrageenan fibers form a gel-like mass agar and possibly did not release the antibacterial compound onto the inoculated soft agar plate. Herein, a different method was assessed.

The Beckman Coulter DU 730 UV/Vis spectrophotometer was used to conduct further antibacterial test. *Listeria innocua* F4248 and *Salmonella* Heidelberg 513 were chosen as the model test microorganisms and TSB with 0.5%yeast extract (TSBYE) was the media used in all further studies. The test microorganisms were grown for approximately 18 h then inoculated at a concentration of 1 x $10^6$ cfu/ml. The controls and experimental groups were added to the appropriate 4 ml glass test tubes of inoculated TSBYE after 2 h of incubation for 37$^o$C. The absorbance of all samples was taken every 2 hours for 12 hours at a wavelength of 600 nm. Some experiments

required longer analysis up to 24 hours. The UV/Vis Spectrophotometer was also used to rapidly screen encapsulation solutions, effect of tween 80 on EO-bacterial interactions, and to monitor the effect of carrageenan-based antibacterial fibers grown in 10% and 50% TSB. Minimum antibacterial activity was shown using the UV/Vis Spectrophotometer. Results are presented with means of duplicate experiments. Cell number calculations were derived to determine the antibacterial activity of the fibers.

The antibacterial activity of the carrageenan-based antibacterial fibers was also evaluated by treating test microorganism with the fibers and monitoring growth by calculating cell numbers over a 12 or 16 hour period. Initially, the test microorganisms were grown for approximately 18 hours then inoculated at a concentration of 1 x $10^6$ cfu/ml. Results in later experiments showed an initial inoculating concentration of 1 x $10^4$ cfu/ml is sufficient for antibacterial test. Controls and experimental groups were added to appropriate tubes containing 4 ml glass test tubes of TSB after 2 hours. Bacterial growth was monitored by enumerating cell numbers after 0, 4, 6, 8, 10, and 12 h of incubation at 37$^o$C. Enumeration was carried out by preparing 10-fold serial dilutions in phosphate buffer saline (PBS) for plating on tryptic soy agar with yeast extract (TSAYE). Plates were incubated for 24 h at 37$^o$C. Results are means of duplicate experiments with four replicates.

### 2.2.7 Scanning Electron Microscopy

*S.* Enteritidis 1344 and *L. monocytogenes* F4244 cells were treated with antibacterial fibers for 8 h. The treated cells were then centrifuged for 4 min at 8000 rpm. The 0.3% TSB was decanted to leave a visible pellet of treated cells. The cells were

fixed in 2.5% glutaraldehyde fixative solution. The cells were then treated with 1% osmium tetroxide for one hour followed by three washes of deionized water. Next, the cells were washed with 50% ethanol, washed twice with 70% ethanol, washed with 85% ethanol, three times with 95% ethanol, and three times with 100% ethanol. Lastly, cells were dried using hexamethyldisilazane. The cells were observed under a Nova NanoSEM ™ 200 series microscope using an accelerating voltage of 50 kV. Images were captured at magnifications of 15,000 and 50, 0000.

# CHAPTER 3. RESULTS & DISCUSSION

## 3.1 Release Profile

In the IC: eugenol fiber release was conducted in deionized water and there was an initial burst of eugenol release during the first 10 mins (Figure 1). The fibers completely released the encapsulated eugenol around 40 mins. IC: carvacrol fibers release studies were conducted in PBS to determine if the antibacterial component is released during the lag phase of bacteria growth. Results indicate the fibers also dissolve in PBS, and gradually release carvacrol in approximately, 40 minutes. It was concluded that the antibacterial molecules could be released in broth-based assays.

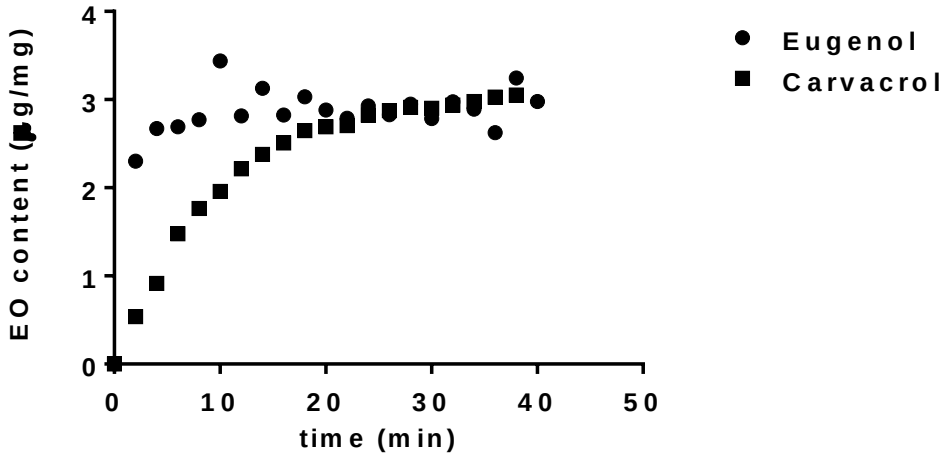



Figure 1. Release Profiles of eugenol and carvacrol encapsulated Iota-carrageenan fibers

## 3.2 Disc Diffusion Assay

In the disc diffusion assay analysis, antibacterial fibers did not create a measureable zone when BHI soft agar was inoculated with *L. innocua* $1x10^7$ cfu/ml, $1x10^6$ cfu/ml, or $1x10^5$ cfu/ml (Figure 2). Fibers seem to absorb water from the soft agar, gel and do not release the eugenol. In order for the maximum antibacterial activity to be demonstrated, the encapsulated eugenol must be released completely. However, when 100 mg of complexed fibers were tested against $1x10^6$ cfu/ml of *L. innocua,* an inhibition of approximately 24.53 mm has been observed.

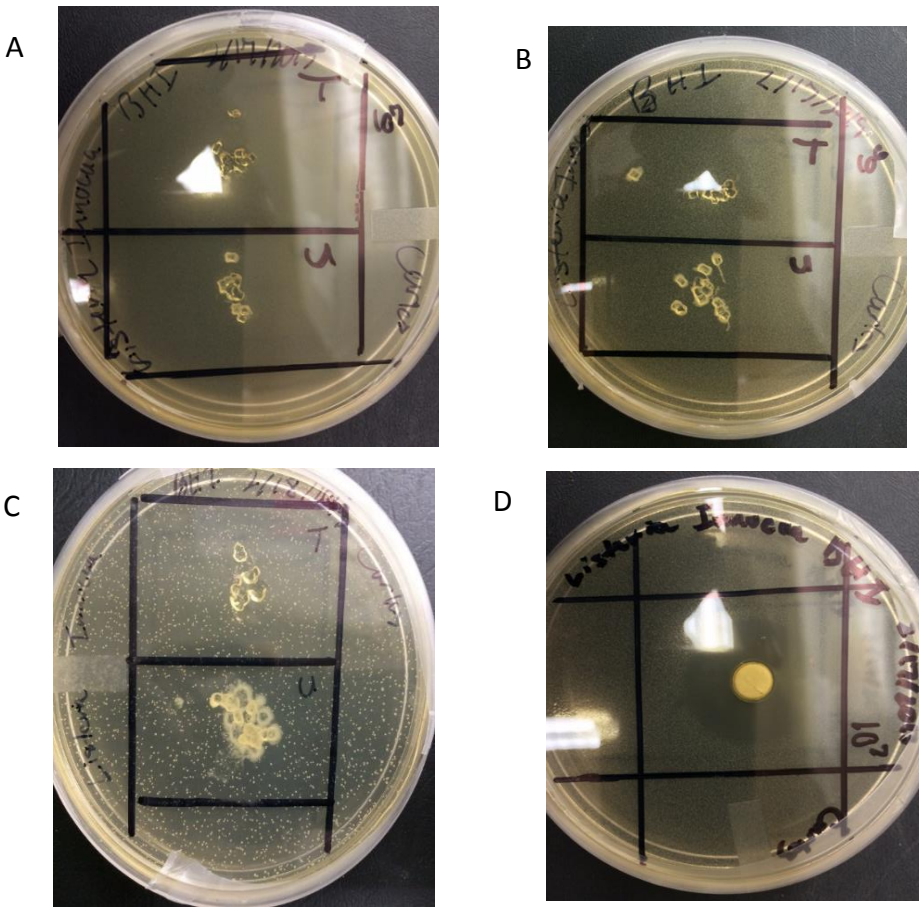



Figure 2. Zone of Inhibition Assay, A. T (iota-carrageenan fibers encapsulated with 47% eugenol: 50% ethanol) and U (iota-carrageenan fibers) tested against $1x10^7$ cfu/ml *L. innocua.* B. T and U tested against $1x10^6$ cfu/ml C. T and U tested against $1x10^5$ cfu/ml D. 5 µl of eugenol tested against $1x10^7$ cfu/ml.

E

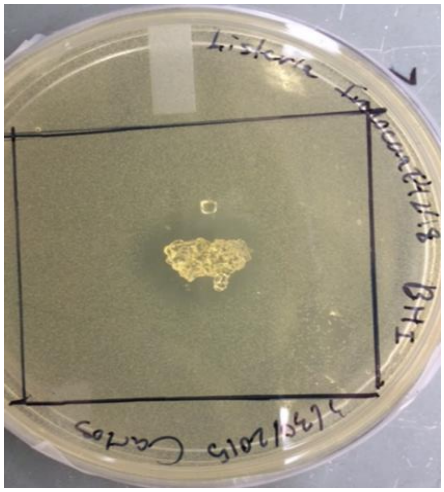


Figure 2. Continued, E. 100 mg of iota-carrageenan fibers encapsulated with 47% eugenol: 50% ethanol tested against $1x10^6$ cfu/ml *L. innocua*

## 3.3 Antibacterial Activity Assay

The antibacterial efficacy was determined using a *UV/Vis Spectrophotometer.* The IC: eugenol fibers were tested against *L. innocua* F4248 and *S*. Heidelberg. During the initial experiments, there was no noticeable reduction in bacteria treated with IC: Eugenol fibers (Figure 3). *L. innocua* treated with non-encapsulated IC fibers also trended with the control for 12 h. As expected, pure Eugenol completely inhibited the growth of *L. innocua* after being added to the test tube. Results were similar when the complexed fibers were tested against *S*. Heidelberg. The *S*. Heidelberg sample treated with IC: Eugenol fibers trended with the control not having an effect on the growth of bacteria. Cell populations increased from $1x10^6$ cfu/ml to $1x10^8$ cfu/ml. Eugenol was also lethal to *S*. Heidelberg; no growth was detected in its tube.

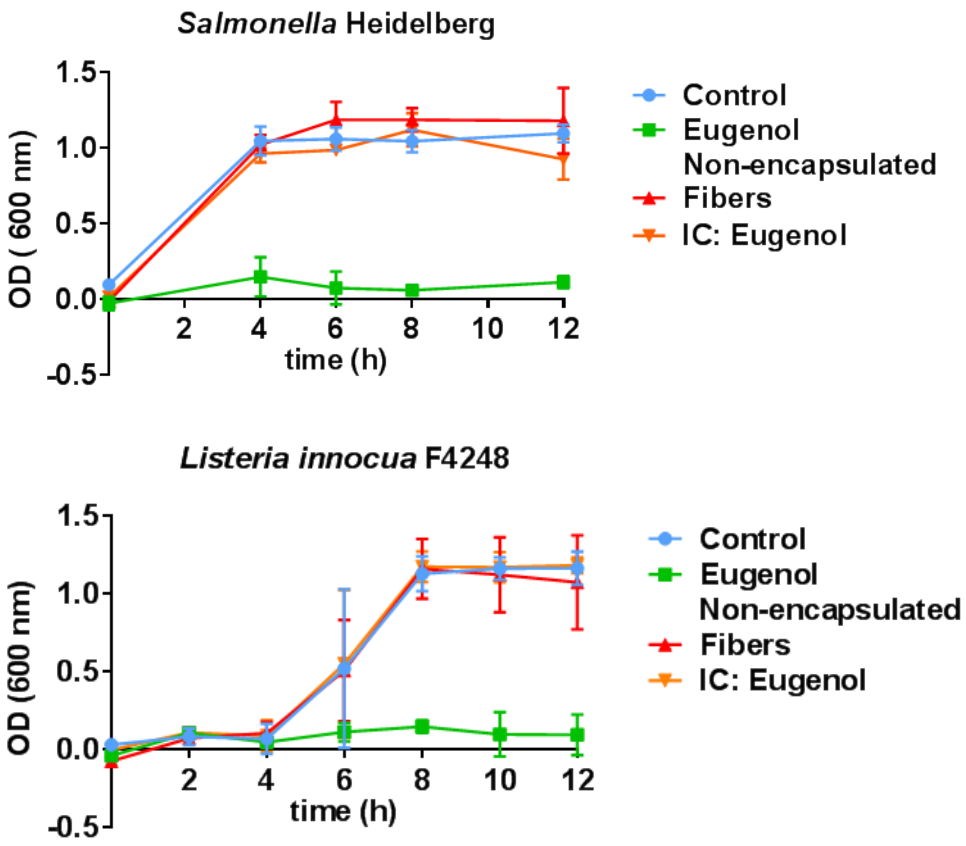



Figure 3. Effect of IC: Eugenol fibers on the growth of bacteria, 1 µl eugenol, Non-encapsulated Fibers- 1 mg iota-carrageenan fibers, IC- 1 mg iota-carrageenan fibers encapsulated with 47% eugenol: 50% ethanol

Carvacrol and Eugenol were mixed with ethanol to determine their efficacy against *S*. Heidelberg. Ethanol did not have an effect on growth of the bacteria (Figure 4). After two hours of incubation time the optical densities (OD) of the negative control and ethanol sample were 0.423 and 0.391, respectively. After 12 hours of incubation time their optical densities were 1.099 and 1.149. No growth was detected in *S*. Heidelberg samples treated with eugenol and carvarol. After 12 hours of incubation time, their OD was 0.055 and 0.075. The 47% carvacrol: 50% ethanol solution was more effective against *S*. Heidelberg than the 47% eugenol: 50% ethanol solution. Optical

densities of samples treated with EE and CE were 0.134 and 0.005 after 2 h, respectively. After 12 h, the OD of samples treated with EE and CE were 0.925 and 0.031, respectively.

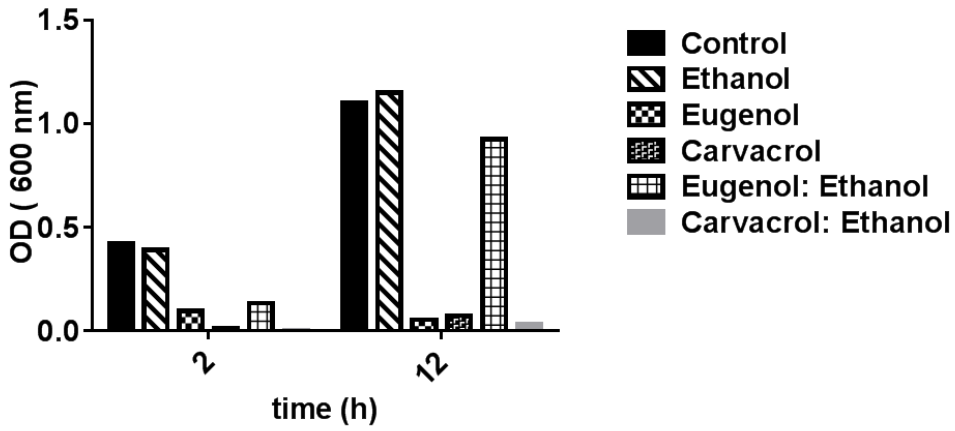



Figure 4. Comparison of eugenol and carvacrol in an antibacterial assay, 2 µl Ethanol, 2 µl Eugenol, 2 µl Carvacrol, EugenolP: Ethanol- 2 µl 47% eugenol: 50% ethanol, Carvacrol: Ethanol- 2 µl 47% carvacrol: 50% ethanol

IC: carvacrol fibers (ICC) were tested against *S*. Heidelberg grown in 1.5 % TSB (50% of normal recipe) and 0.3% TSB media (10% of normal recipe). Results showed ICC were not effective against *S*. Heidelberg in 1.5 TSB for 12 h (Figure 5). However, there was a slight decrease in cell populations treated with ICC, but not significant reduction after 6 hours of incubation. Non- Encapsulated did not have an effect on *S*. Heidelberg cell populations but the Carvacol: Ethanol (CE) solution completely inhibited growth as expected. Results were different when ICC fibers were tested against *S*. Heidelberg in 0.3% TSB. ICC fibers reduced cell populations after 6 h of, but cell numbers increased 8 h of incubation time. Interestingly, after 12 h the OD of the ICC treated sample was significantly lower than the negative control.

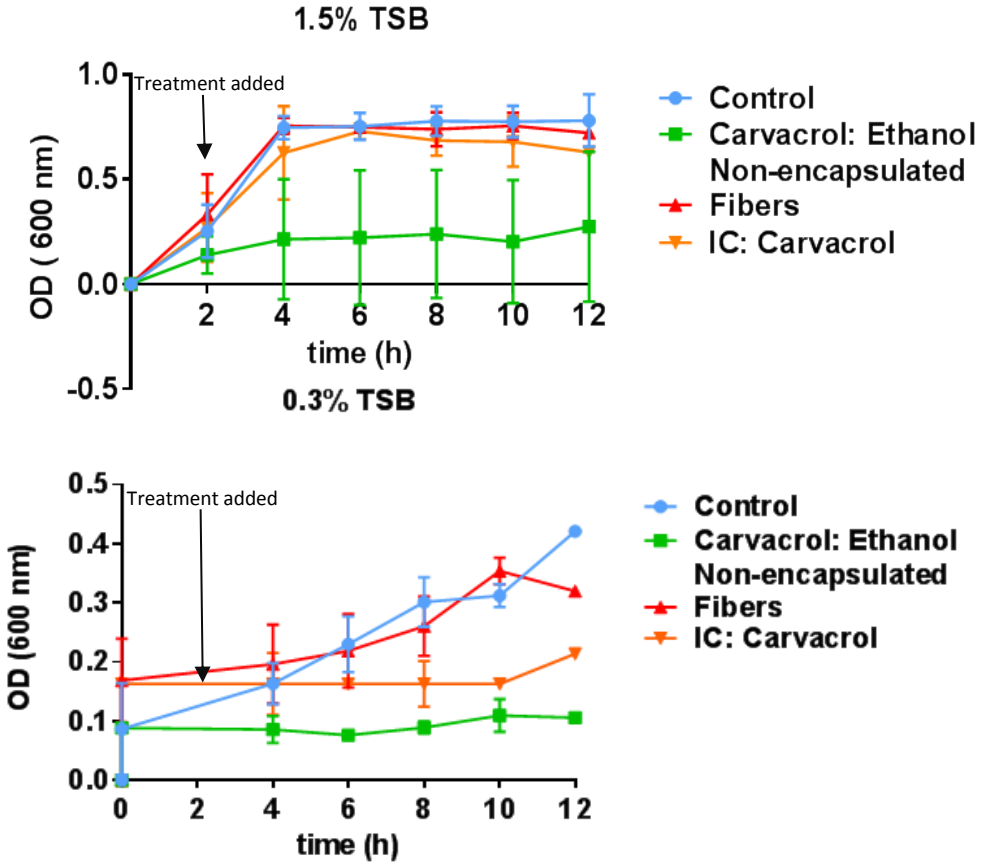



Figure 5. Effect of IC: Carvacrol fibers on the growth of bacteria in reduced nutrient broth, Carvacrol: Ethanol- 2 µl 47% carvacrol: 50% ethanol, Non-encapsulated Fibers- 5 mg iota-carrageenan fibers, IC: Carvacrol- 5 mg iota-carrageenan fibers encapsulated with 47% carvacrol: 50% ethanol

The effect of fibers encapsulated with carvacrol: Tween 80 at various concentrations was compared to fibers encapsulated with 49% carvacrol: 50% ethanol (Figure 6). All fiber samples were effective at reducing cell populations *S*. Heidelberg in 0.3% TSB. Fibers encapsulated with 50% carvacrol: 50% Tween 80 was the most effective against *S*. Hiedelberg and was comparable to positive control, Carvacrol: Ethanol After 16 h, the OD of the samples treated with CE and C 50%: T80 50%-F were 0.1508 and 0.2298 respectively. However, fibers encapsulated with CE were the least effective. After 16 h the OD of the samples was 0.3752.

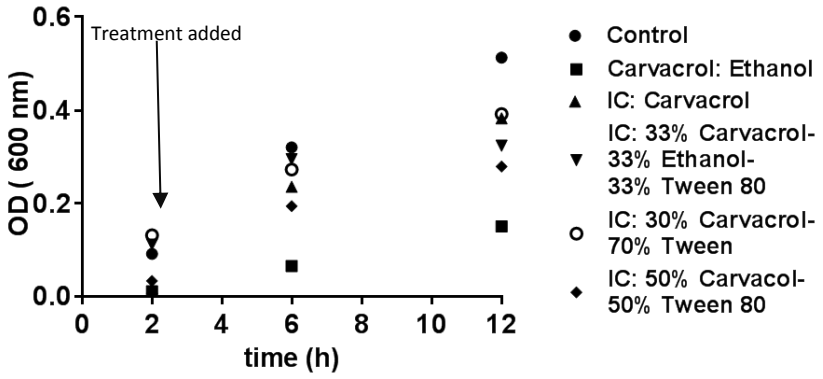



Figure 6. Comparison of various IC fibers in an antibacterial assay, Carvacrol: Ethanol- 1 µl 47% carvacrol 50% ethanol, IC: Carvacrol- 5 mg iota-carrageenan fibers encapsulated with 47% carvacrol: 50% ethanol, IC: 33% Carvacrol- 33% Ethanol- 33% Tween 80- 5 mg iota-carrageenan fibers encapsulated with 33% carvacrol: 33% ethanol: 33% Tween 80, IC: 50% Carvacrol-50% Tween 80- 5 mg iota-carrageenan fibers encapsulated with 50% carvacrol: 50% Tween 80, IC: 30% Carvacrol- 70% Tween 80-5 mg iota-carrageenan fibers encapsulated with 30% carvacrol: 70% Tween 80

IC: 50% Carvacrol-50% Tween 80 (ICCT) fibers were tested against, *E. coli* O157: H7 ATCC 43295, *S*. Enteritidis ENT 1344, *S*. Heidelberg 513, *S. aureus*, *L. innocua* F4248, and *L. mono* F4244 (Figure 7 and 8). Results showed ICCT fibers were effective against most bacterial strains. When *S*. Enteritidis was treated with ICCT fibers, cell populations were comparable to the control, but later slightly reduced after 8h, 10 h, and 12 h, of incubation. ICCT fibers extended the lag phase of *S.* Heidelberg and resulted in a 1-log reduction. There was a 1-log difference between the control and ICCT fiber treated sample for 12 h. Cell populations of *E. coli* O157: H7 treated with ICCT fibers were decreased for 10 h and increased after 12 h of incubation time.

IC: 50% Carvacrol-50% Tween 80 fibers were not effective against *S. aureus.* When *S. aureus* was treated with ICCT fibers, cell populations were comparable to the control for 12 h. The ICCT fibers were most effective against *L. innocua* F4248, and *L. mono* F4244. In the *L. innocua* test, ICCT fibers reduced cell populations by 4-logs

compared to the control after 12 h. Similarly, in the *L. monocytogenes* test, ICCT fibers reduced cell populations by 3-logs after 12 h of incubation time. Interestingly, the *L. monocytogenes* treated with ICCT fibers increased to 1 x $10^5$ cfu/ml after 4 h of incubation, but later decreased back to 1 x $10^4$ cfu/ml for the remaining time intervals of the assay.

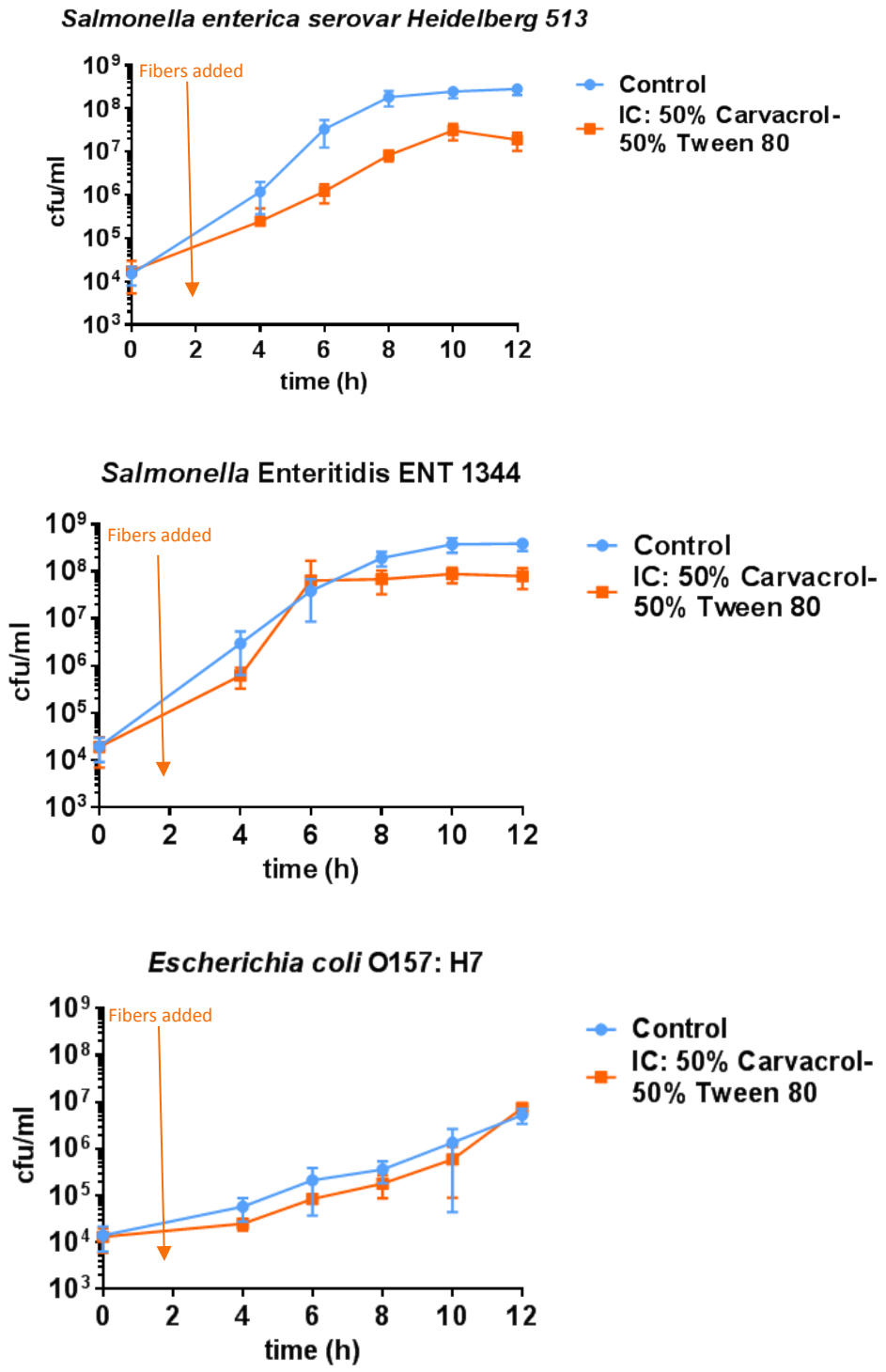



Figure 7. Effect of antibacterial fibers on gram-negative bacteria, IC: 50% Carvacrol-50% Tween 80- 10 mg iota-carrageenan fibers encapsulated with 50% carvacrol: 50% Tween 80

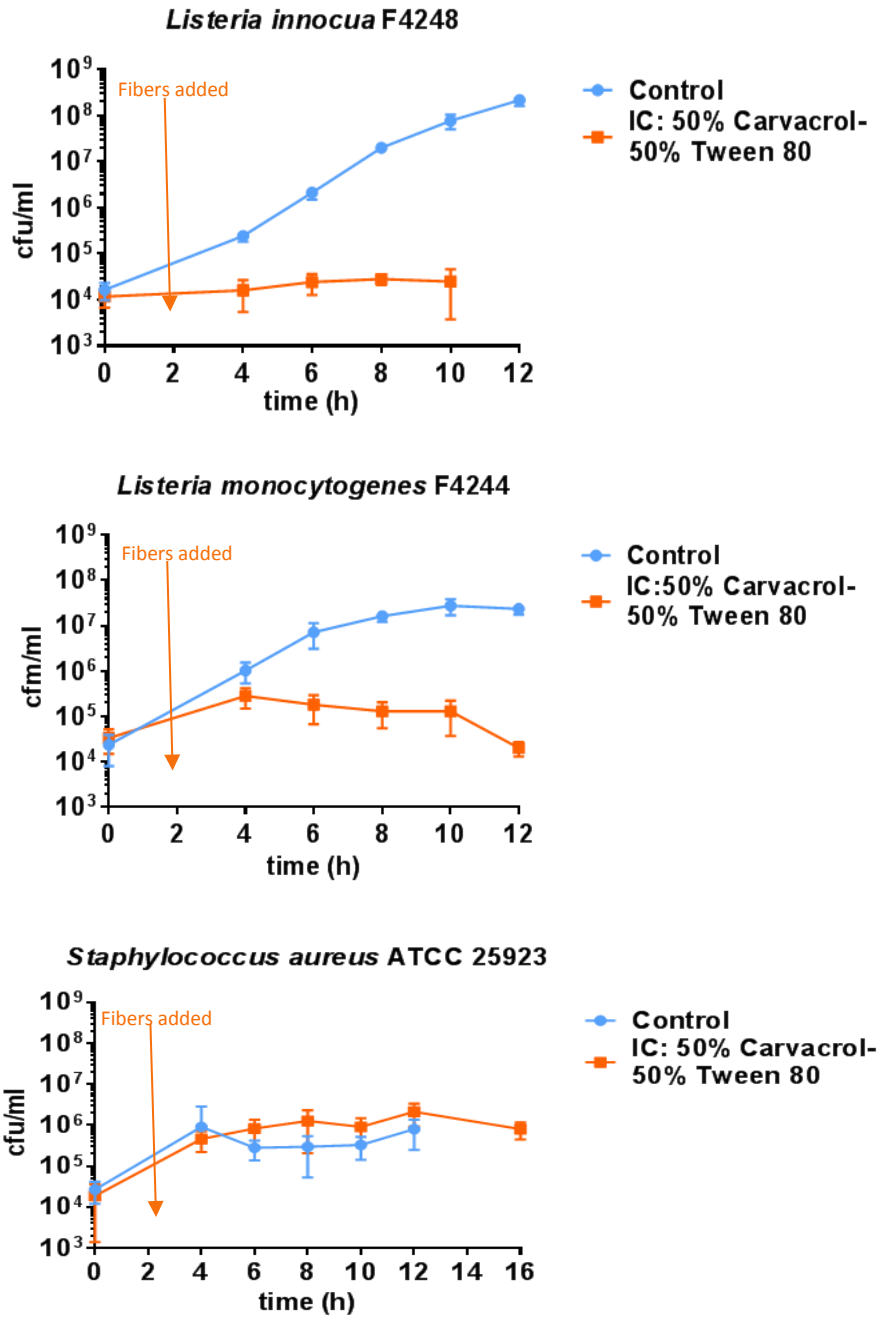



Figure 8. Effect of antibacterial fibers on gram-positive bacteria, IC: 50% Carvacrol-50% Tween 80- 10 mg iota-carrageenan fibers encapsulated with 50% carvacrol: 50% Tween 80

## 3.4 Scanning Electron Microscopy (SEM)

SEM was carried out using a Nova NanoSEM ™ 200 series microscope. Images of IC fibers, and IC fibers encapsulated with 50% Carvacrol: 50 % Tween 80 were captured (Figure 9). IC fibers appeared light and were composed of flaky crystals on the surface. The average diameters of non-encapsulated fibers were 188.35 µm ± 59.85. However, IC fibers encapsulated with 50% Carvacrol: 50 % Tween 80 had smooth surfaces and average diameter increased to 204.70 µm ± 58. 05.

SEM images were also captured for the test microorganisms *Salmonella* Heidelberg and *L. monocytogenes*, grown in the presence of IC 50%C: 50%T80 fibers. The images revealed the *S.* Heidelberg and *L. monocytogenes* growth controls had general characteristics of the respective bacteria and appeared healthy. *S*. Heidelberg treated with IC 50%C: 50%T80 fibers were not affected by the antibacterial compound. The *Salmonella* aggregated into large clusters. *L. monocytogenes* treated with IC 50%C: 50%T80 fibers were damaged. While some cells appeared healthy and unaffected, others were transparent and dense (Figure 9, E&F).

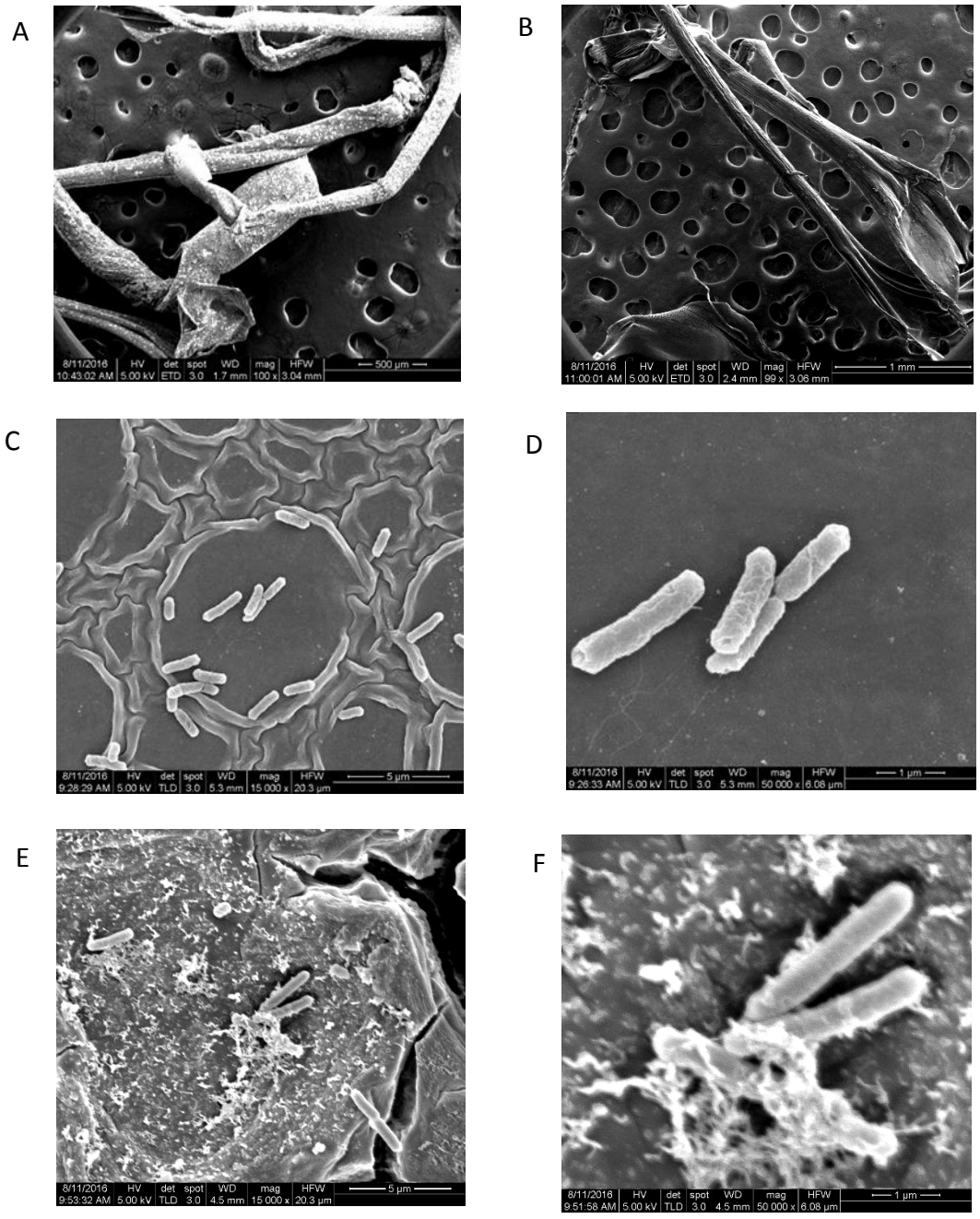
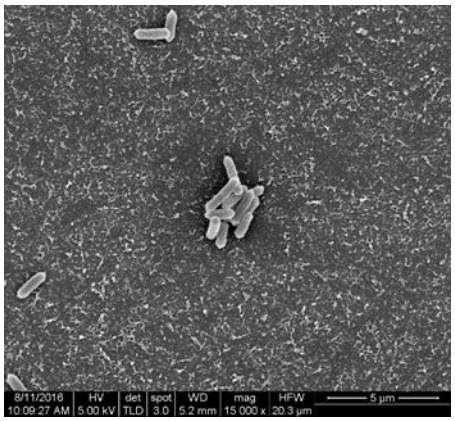
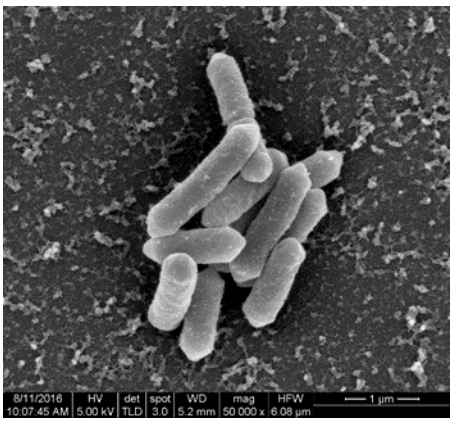



Figure 9. Scanning Electron Microscopy images of fibers, *Salmonella* Enteritidis and *Listeria monocytogenes* treated with antibacterial fibers A. iota-carrageenan fibers, B. iota-carrageenan fibers encapsulated with 50% carvacrol: 50% Tween 80, C & D. *S*. Enteritidis growth control captured at a magnification of 15, 000 (low magnification) and 50, 000 (high magnification) respectively, E & F. *S*. Enteritidis treated with antibacterial fibers captured at a low and high magnification,

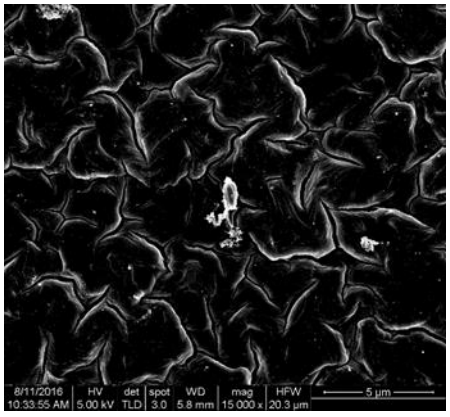
G




H




I




J

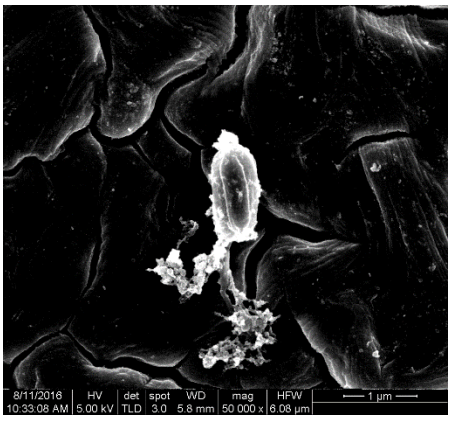



Figure 9 Continued. G & H. *L. monocytogenes* growth control captured at a low and high magnification, I & J. *L. monocytogenes* captured at a low and high magnification

## 3.5 Discussion

Herein, it has been demonstrated that iota-carrageenan fibers could be fabricated and encapsulated with single components of essential oils. The fibers were tested against several pathogenic bacterial species and it was discovered that the complex fibers could indeed reduce the proliferation of tested microorganisms. Release studies were conducted on two types of antibacterial fibers, IC 49% eugenol: 50% ethanol and IC 50% carvacrol: 50% Tween 80. The antibacterial fibers are quickly saturated, releasing the EO components in approximately 40 mins. Fibers containing eugenol and ethanol released the antibacterial compound with a quick burst while the carvacrol was released from the fibers gradually over time. The differences in release rate could be attributed to the encapsulated ethanol and Tween 80. Both ethanol and Tween 80 are miscible in water, but Tween 80 is a larger molecule than ethanol. Both types of fibers encapsulated approximately the same amount of the EO components. Initially, eugenol and ethanol were used as the antibacterial encapsulation solution for fibers, but the antibacterial effect was not significant. It was later determined that carvacrol has a lower minimum inhibitory concentration than eugenol (Burt, 2004). Our study clearly suggests that in an antibacterial assay, carvacrol mixed with ethanol was a more effective encapsulation solution than eugenol: ethanol. During our study, it was suggested; nutrients in broth could interfere with the antibacterial effect of fibers encapsulated with essential oil components. Results in Fig 6. suggest, antibacterial fibers can reduce the growth of bacteria in 1.5 TSB % and 0.3 TSB % (0.50% and 10% original TSB composition, respectively).

Initially, fibers were dried of the encapsulation solution overnight and treated to the test microorganisms in the beginning of the assay the following day (Fig 3 & 4). Due to the volatility of essential oils, some of the antibacterial effect was lost. For the purpose of optimization, fibers were dried for two hours and treated to the test microorganisms two hours after inoculation of bacteria (Fig 5-8). We also showed Tween 80 serves as a viable surfactant in Iota-carrageenan fibers. Herein, IC 50% carvacrol: 50% Tween 80 fibers were used to be tested against pathogenic bacteria (Fig 6).

Our antibacterial compounds reduced the growth of *L. monocytogenes* and *L. innocua*, but were not effective when tested against *Salmonella* and *E. coli*. Similar results were reported by *Krigel et al* (2009, 2010) when they electrospun fibers containing eugenol, surfactant solution Surfynol 465®, and poly (vinyl alcohol). These fibers were tested against two stains of *L. monocytogenes* and *Salmonella* Typhimurium. Cell populations in both bacteria species were reduced for 12 hours. However, cell populations increased *Salmonella* Typhimurium 2486 increased comparable to the growth control after 30 hours. Interestingly, cell populations were decreased tremendously after 30 hours in assay with *Salmonella* Typhimurium 2576. Some researchers have reported Gram-positives to be more susceptible to the action of EOs and EO components. The claim cannot be made in the case of our iota-carrageenan-EO complexes as *Salmonella* and *E. coli* (Gram-negative) were more susceptible than *Staphylococcus aureus* (Gram-positive). Gram-negative cells possess an outer membrane surrounding their cell wall, which could restrict diffusion of hydrophilic compounds

through its lipopolysaccharide layer. However, EOs are capable of disintegrating through the outer membrane layer and increasing permeability in the cytoplasmic membrane. Perhaps the microorganism's response to the mode of action of antibacterial compounds is genetic or they could be resistant. Organisms may acquire genes encoding enzymes, such as β-lactamases, that destroy the antibacterial agent before it takes effect. Bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site. Bacteria can also acquire several genes for a metabolic pathway that alters the cell wall that no longer contains the binding sites of the antimicrobial agents (Tenover, 2006). However, there is no scientific evidence of EO resistance due to genetics. This may be of scientific interest in the future.

Tween 80 was a more effective surfactant than ethanol in terms of the antibacterial activity of the fibers. It should be investigated how other surfactants could affect the loading and release nature of essential oils from the polysaccharide fibers. Various balancing ions could also be paired with iota-carrageenan, namely calcium, magnesium and potassium, to name a few and have an effect on the loading amount and mechanism. Combinations of EO components have reportedly had synergistic effects when tested against bacteria. EO components have also had synergetic effects with food preservatives (Burt, 2004). This should be considered in further studies with polysaccharide fibers. Encapsulation of essential oils into antibacterial polysaccharides should also be considered in further studies.

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APPENDICES

## Appendix A Additional Results of Antibacterial Activity Assays

Appendix A contains results from trial antibacterial assays that contributed to the foundation of our methodology to demonstrate the antibacterial activity of well-organized iota-carrageenan fibers encapsulated with essential oil components. Figure A.1. displays results from an assay conducted to determine if 0.01% Tween 80 had an effect on the growth of bacteria when it is added to tryptic soy broth with yeast extract (TSBYE) at various times intervals or premixed in TSBYE. Results showed, when 0.01% Tween 80 is added to TSBYE after 0, 2, and 4 h of inoculation, it does not have an effect on the growth of bacteria. When 0.01% Tween is premixed with TSBYE, it slightly reduced the proliferation of *Listeria innocua* F4248 after 6 hours. Figure A.2. contains results from an assay conducted to determine if the most viable time treat bacteria with IC: eugenol fibers. Eugenol (1µl) was a positive control. Results showed that IC: eugenol fibers did not have an effect on the growth of *Listeria innocua* F4248 when treated after 0, 2, or 4 h after inoculation. Figure A.3. displays results for an assay conducted to determine the efficacy of various essential oils encapsulated in iota-carrageenan fibers and tested against *Listeria innocua* F4248. IC fibers encapsulated with 47% eugenol, carvacrol, or trans-cinnamaldehyde did not show major differences in levels of efficacy. Carvacrol (1µl) inhibited the growth of *L. innocua* F4248 significantly. Figure A.4. dsiplays results from an experiment conducted to compare IC fibers containing 0.1% Tween 80,

encapsulated with various concentrations of carvacrol, and tested against *Salmonella enterica* serovar Heidelberg 513. Results showed fibers at effective at several concentrations (10-100%). IC fibers containing 0.1% Tween 80 and encapsulated with 75% and 100% carvacrol were most effective against *Salmonella* Heidelberg.

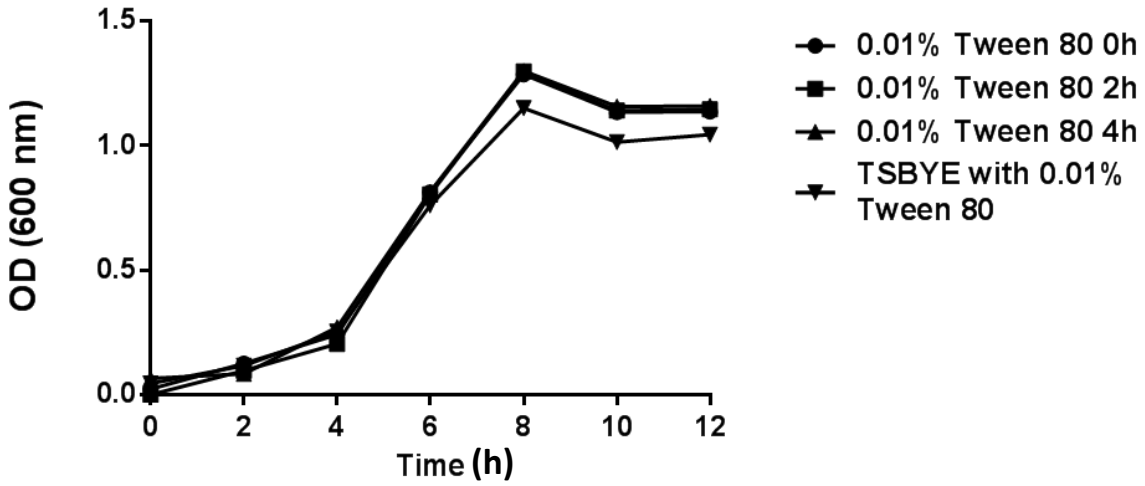


Figure A.1. Effect of Tween 80 on the growth of *Listeria innocua* F4248

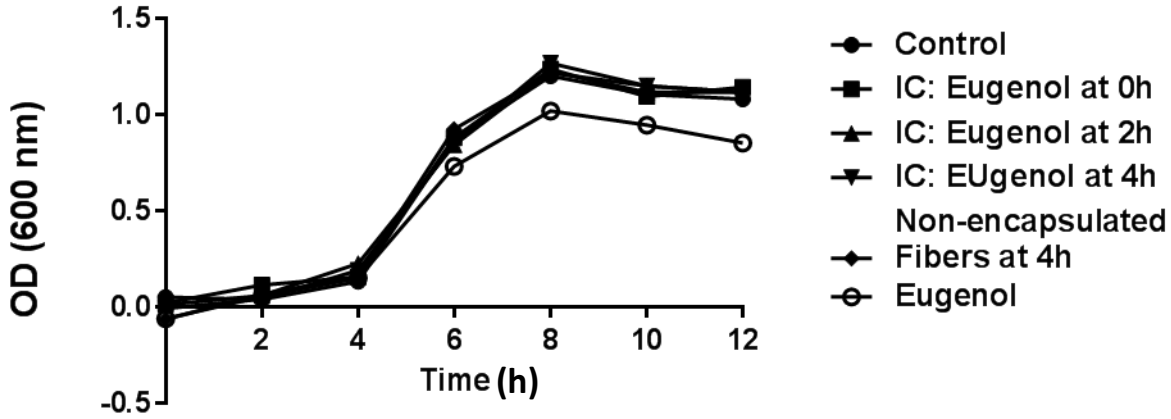


Figure A.2. *Listeria innocua* F4248 treated with IC: Eugenol fibers at different time intervals

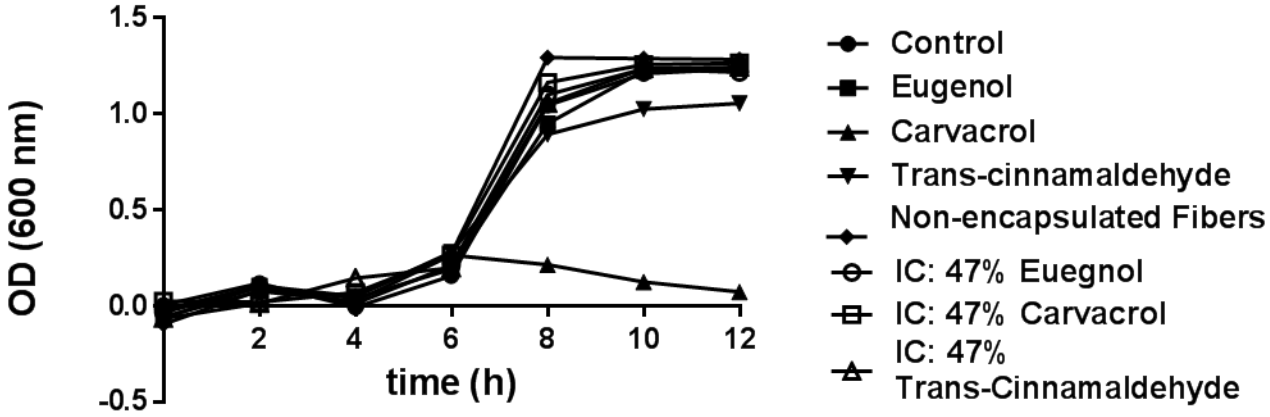


Figure A.3. Comparison of IC fibers encapsulated with various essential oil components on the growth of *Listeria innocua* F4248

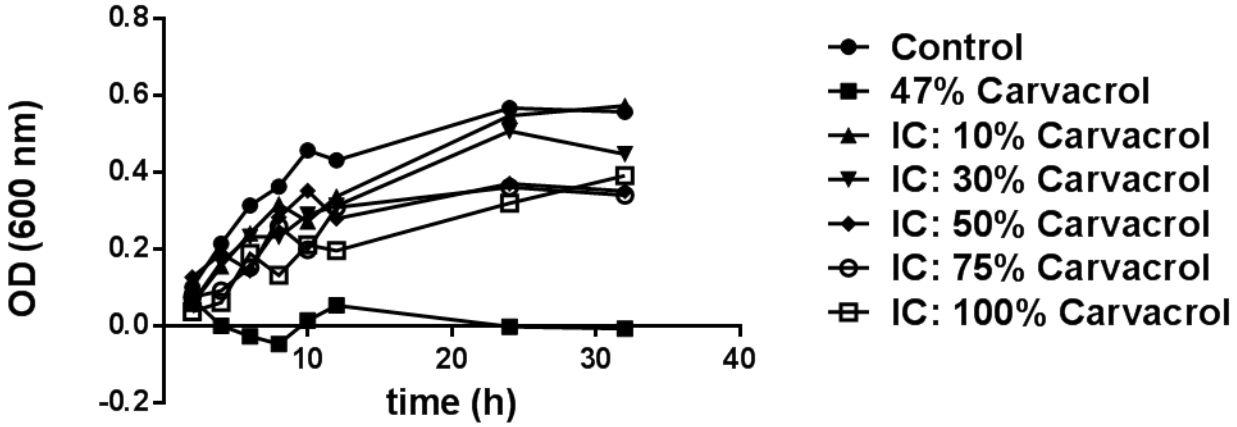


Figure A.4. Comparison of IC fibers encapsulated with carvacrol at various concentrations and treated to *Salmonella* Heidelberg

## Appendix B Raw Data

Appendix B contains raw data from results displayed in Chapter 3 (Table B.1-12).

Table B. 1. *Eugenol and carvacrol release from IC fibers and encapsulated amount*

| *Time (min)* | *Eugenol (ug/mg)* | *Carvacrol (ug/mg)* |
|---|---|---|
| *0* | 0 | 0 |
| *2* | 2.301673 | 0.539125 |
| *4* | 2.671729 | 0.915995 |
| *6* | 2.691003 | 1.476985 |
| *8* | 2.770025 | 1.764672 |
| *10* | 3.438825 | 1.957422 |
| *12* | 2.814355 | 2.216341 |
| *14* | 3.128517 | 2.377445 |
| *16* | 2.823992 | 2.506904 |
| *18* | 3.030221 | 2.647871 |
| *20* | 2.881813 | 2.691024 |
| *22* | 2.787372 | 2.705409 |
| *24* | 2.929998 | 2.826237 |
| *26* | 2.829774 | 2.875144 |
| *28* | 2.949271 | 2.912543 |
| *30* | 2.785444 | 2.901036 |
| *32* | 2.976255 | 2.938435 |
| *34* | 2.89145 | 2.978711 |
| *36* | 2.625472 | 3.027618 |
| *38* | 3.246087 | 3.050633 |
| *40* | 2.978182 | |

Table B. 2. *Effect of IC: Eugenol Fibers on the growth Salmonella enterica serovar Heidelberg 513*

| *Time (h)* | *Control* | *Non-encapsulated FIbers* | *IC: Eugenol FIbers* | *Eugenol* |
|---|---|---|---|---|
| | OD (600nm) | | | |
| *0* | 0.098± 0.012 | -0.012± 0.018 | 0.016± 0.011 | -0.025± 0.045 |
| *4* | 1.047± 0.096 | 1.023± 0.065 | 0.964± 0.060 | 0.149± 0.130 |
| *6* | 1.060± 0.077 | 1.187± 0.119 | 0.987± 0.004 | 0.076± 0.108 |
| *8* | 1.046± 0.074 | 1.187± 0.076 | 1.120± 0.111 | 0.062± 0.012 |
| *12* | 1.098± 0.059 | 1.180± 0.217 | 0.927± 0.136 | 0.114± 0.041 |

Table B. 3. *Effect of IC: Eugenol Fibers on the growth Listeria innocua F4248*

| *Time (h)* | *Control* | | *Non-encapsulated FIbers* | | *IC-Eugenol FIbers* | | *Eugenol* | |
|---|---|---|---|---|---|---|---|---|
| | OD (600nm) | | | | | | | |
| *0* | 0.030± | 0.034 | -0.076± | 0.026 | -0.004± | 0.041 | -0.040 | 0.029 |
| *2* | 0.081± | 0.054 | 0.071± | 0.018 | 0.107± | 0.014 | 0.104 | 0.002 |
| *4* | 0.068± | 0.096 | 0.104± | 0.072 | 0.087± | 0.103 | 0.046 | 0.007 |
| *6* | 0.517± | 0.508 | 0.503± | 0.326 | 0.547± | 0.475 | 0.110 | 0.062 |
| *8* | 1.125± | 0.112 | 1.158± | 0.192 | 1.170± | 0.099 | 0.146 | 0.041 |
| *12* | 1.160± | 0.072 | 1.119± | 0.241 | 1.167± | 0.097 | 0.096 | 0.141 |

Table B. 4 *Comparison of eugenol and carvacrol in an antibacterial assay against Salmonella Heidelberg*

| *Treatment* | *2 h* | *12 h* |
|---|---|---|
| | OD (600 nm) | |
| *Control* | 0.423 | 1.100 |
| *Ethanol* | 0.391 | 1.150 |
| *Eugenol* | 0.0977 | 0.0548 |
| *47% Eugenol: 50% Ethanol* | 0.134 | 0.926 |
| *47% Carvacrol: Ethanol* | 0.0045 | 0.0316 |

Table B. 5 *Effect of IC: Carvacrol fibers on the growth of Salmonella Heidelberg in 1.5% tryptic soy broth*

| *Time (h)* | *Control* | *47% Carvacrol: 50% Ethanol* | *Non-encapsulated Fibers* | *IC: Carvacrol* |
|---|---|---|---|---|
| | (600nm) | | | |
| *0* | 0 | 0 | 0 | 0 |
| *2* | 0.253± 0.126 | 0.140± 0.089 | 0.333± 0.191 | 0.271± 0.164 |
| *4* | 0.747± 0.055 | 0.214± 0.286 | 0.756± 0.039 | 0.627± 0.223 |
| *6* | 0.753± 0.065 | 0.223± 0.322 | 0.749± 0.028 | 0.729± 0.038 |
| *8* | 0.778± 0.070 | 0.240± 0.306 | 0.739± 0.082 | 0.686± 0.073 |
| *10* | 0.775± 0.076 | 0.204± 0.294 | 0.755± 0.061 | 0.680± 0.119 |
| *12* | 0.780± 0.125 | 0.274± 0.358 | 0.722 | 0.629± 0.011 |

Table B. 6 *Effect of IC: Carvacrol fibers on the growth of Salmonella Heidelberg in 0.3% tryptic soy broth*

| *Time (h)* | *Control* | | *47% Carvacrol: 50% Ethanol* | | *Non-encapsulated Fibers* | | *IC: Carvacrol* | |
|---|---|---|---|---|---|---|---|---|
| | (600nm) | | | | | | | |
| *0* | 0 | | 0 | | 0 | | 0 | |
| *2* | 0.087± | 0.077 | 0.089± | 0.001 | 0.169± | 0.071 | 0.163± | 0.0001 |
| *4* | 0.163± | 0.035 | 0.286± | 0.022 | 0.196± | 0.067 | 0.163± | 0.052 |
| *6* | 0.230± | 0.047 | 0.322± | 0.001 | 0.219± | 0.063 | 0.163± | 0.004 |
| *8* | 0.301± | 0.042 | 0.306± | 0.001 | 0.261± | 0.050 | 0.163± | 0.039 |
| *10* | 0.312± | 0.020 | 0.294± | 0.028 | 0.354± | 0.023 | 0.163± | 0.005 |
| *12* | 0.42 | | 0.35 | | 0.32 | | 0.21 | |

Table B. 7. *Effect of antibacterial fibers on Salmonella Enteritidis ENT 1344*

| *Time (h)* | Control | IC: 50% Carvacrol: 50% Tween 80 |
|---|---|---|
| | cfu/ml | |
| *0* | 1.943E+04± 1.034E+04 | 1.886E+04± 1.195E+04 |
| *4* | 2.985E+06± 2.350E+06 | 6.167E+05± 2.881E+05 |
| *6* | 3.871E+07± 3.005E+07 | 6.385E+07± 1.043E+08 |
| *8* | 1.943E+08± 6.680E+07 | 6.850E+07± 3.548E+07 |
| *10* | 3.800E+08± 1.310E+08 | 8.900E+07± 3.252E+07 |
| *12* | 3.929E+08± 1.186E+08 | 8.000E+07± 3.763E+07 |

Table B. 8. *Effect of antibacterial fibers on Salmonella enterica serovar Heidelberg 513*

| *Time (h)* | *Control* | *IC: 50% Carvacrol: 50% Tween 80* |
|---|---|---|
| | cfu/ml | |
| *0* | 1.500E+04± 6.904E+03 | 1.771E+04± 1.242E+04 |
| *4* | 1.186E+06± 8.295E+05 | 2.475E+05± 2.473E+05 |
| *6* | 3.338E+07± 2.087E+07 | 1.208E+06± 5.665E+05 |
| *8* | 1.829E+08± 7.111E+07 | 8.350E+06± 2.333E+06 |
| *10* | 2.457E+08± 7.300E+07 | 3.100E+07± 1.273E+07 |
| *12* | 2.825E+08± 8.049E+07 | 1.900E+07± 8.544E+06 |

Table B. 9. *Effect of antibacterial fibers on Escherichia coli O157: H7 ATCC 43295*

| *Time (h)* | *Control* | *IC: 50% Carvacrol: 50% Tween 80* |
|---|---|---|
| | cfu/ml | |
| *0* | 1.414E+04± 7.734E+03 | 1.313E+04± 7.080E+03 |
| *4* | 5.800E+04± 3.033E+04 | 2.500E+04± 7.071E+03 |
| *6* | 2.117E+05± 1.745E+05 | 8.500E+04± 1.732E+04 |
| *8* | 3.620E+05± 1.795E+05 | 1.800E+05± 9.274E+04 |
| *10* | 1.349E+06± 1.306E+06 | 5.950E+05± 5.049E+05 |
| *12* | 5.300E+06± 1.901E+06 | 7.450E+06± 1.909E+06 |

Table B. 10. *Effect of antibacterial fibers on Listeria innocua F4248*

| *Time (h)* | *Control* | | *IC: 50% Carvacrol: 50% Tween 80* | |
|---|---|---|---|---|
| | cfu/ml | | | |
| *0* | 1.650E+04± | 6.714E+03 | 1.188E+04± | 5.083E+03 |
| *4* | 2.430E+05± | 5.962E+04 | 1.617E+04± | 1.070E+04 |
| *6* | 2.115E+06± | 6.063E+05 | 2.450E+04± | 1.168E+04 |
| *8* | 2.000E+0 | | 2.850E+04± | 7.550E+03 |
| *10* | 7.720E+07± | 2.687E+07 | 2.500E+04± | 2.121E+04 |
| *12* | 2.175E+08± | 5.679E+07 | 1.188E+04± | 5.083E+03 |

Table B. 11. *Effect of antibacterial fibers on Listeria monocytogenes F4244*

| *Time (h)* | *Control* | *IC: 50% Carvacrol: 50% Tween 80* |
|---|---|---|
| | cfu/ml | |
| *0* | 2.620E+04± 1.566E+04 | 2.286E+04± 1.842E+04 |
| *4* | 6.675E+05± 5.000E+05 | 2.000E+05± 1.358E+05 |
| *6* | 4.606E+06± 4.130E+06 | 1.000E+05± 1.131E+05 |
| *8* | 1.338E+07± 4.106E+06 | 1.300E+05± 7.550E+04 |
| *10* | 2.617E+07± 1.063E+07 | 9.667E+04± 9.292E+04 |
| *12* | 2.100E+07± 5.944E+06 | 1.500E+04± 7.071E+03 |

Table B. 12. *Effect of antibacterial fibers on Staphylococcus aureus ATCC 25923*

| *Time (h)* | *Control* | *IC: 50% Carvacrol: 50% Tween 80* |
|---|---|---|
| | cfu/ml | |
| *0* | 2.667E+04± 1.481E+04 | 1.871E+04± 1.729E+04 |
| *4* | 9.100E+05± 1.919E+06 | 4.613E+05± 2.405E+05 |
| *6* | 2.788E+05± 1.425E+05 | 8.250E+05± 5.315E+05 |
| *8* | 2.980E+05± 2.450E+05 | 1.263E+06± 1.054E+06 |
| *10* | 3.275E+05± 1.854E+05 | 9.100E+05± 5.682E+05 |
| *12* | 8.017E+05± 5.552E+05 | 2.123E+06± 1.202E+06 |
| *16* | | 8.000E+05± 3.536E+05 |

## Appendix C Media Composition

Appendix C contains the composition of the different media used in this study. Vendors and lot numbers of chemicals are located in chapter 2.

Table C. 1. *Brain Heart Infusion Broth (1 liter)*

| |
|---|
| 17.5 g (BHI) |
| 1000 ml deionized water |

Table C. 2 *Brain Heart Infusion Agar (1 liter)*

| |
|---|
| 17.5 g (BHI) |
| 8 g agar |
| 1000 ml deionized water |

Table C. 3. *Tryptic Soy Broth with Yeast Extract (1liter)*

| |
|---|
| 30 g (TSB) |
| 5 g yeast extract |
| 1000 ml deionized water |

Table C. 4. *Tryptic Soy Agar with Yeast Extract (1liter)*

| |
|---|
| 30 g (TSB) |
| 5 g yeast extract |
| 8 g agar |
| 1000 ml deionized water |

Table C. 5. *1.5% Tryptic Soy Broth (1 liter)*

| |
|---|
| 15 g (TSB) |
| 1000 ml deionized water |

Table C. 6. *0.3% Tryptic Soy Broth*

| |
|---|
| 3 g (TSB) |
| 1000 ml deionized water |

## Appendix D Microorganism Growth Conditions

Brain heart infusion (BHI) media was used to grow *L. innocua* F4248 for disc diffusion assay. One loop of *L. innocua* was taken from a frozen stock and inoculated into 4 ml of BHI. The inoculated test tube was incubated at 37°C for approximately 18 h. Tryptic soy broth with yeast extract (TSBYE) was used to cultivate all microorganisms in antibacterial activity assays in the study. Subcultures of the test microorganism were prepared by streaking one loop onto tryptic soy agar plates with yeast extract. The streaked plates were incubated at 37°C for 24 h. One single colony from the subculture plate was inoculated into 4 ml of TSBYE. The inoculated tube was incubated at 37°C for approximately.

VITA

# VITA

Carlos Carter was born in Washington D.C., on December 19, 1991. His family moved to Warrenton, NC when he was eleven years old. After graduating from Warren County High School in 2010, he went on to pursue a B.S. in Food & Nutritional Sciences at North Carolina Agricultural and Technical State University in Greensboro, NC. After receiving his B.S., in 2014, he furthered his education at Purdue University in achieving a M.S., in Food Science in West Lafayette, IN. He started his professional career as a Food Scientist with Cargill, Inc. working with the Snacks & Cereal, Food Applications Teams.