Inhibition of Single and Mixed Species Biofilms Formed by *Listeria monocytogenes* and Food Related Bacteria by Natural Compounds

by

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Table of Contents

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Pathogenic bacteria, such as *Listeria monocytogenes*, are easily transferred from biofilms on processing equipment to foods, causing many foodborne outbreaks and illnesses. The antimicrobial and anti-biofilm effects of natural compounds, thymol, carvacrol, trans-cinnamaldehyde and lemongrass essential oil were tested against single and mixed species biofilms of *Listeria monocytogenes, Salmonella enterica* Typhimurium*, Staphylococcus aureus*, *Pseudomonas fluorescens* and *Shewanella baltica.* All natural antimicrobials inhibited single and mixed species biofilm formation and planktonic growth, however, biofilm removal depended on bacteria, antimicrobial, maturity of the biofilm and mixed species interactions. Generally, biofilm cells showed higher resistance than planktonic cells. Bacterial survival in mixed species mostly decreased compared to single species biofilm. Bacterial proportions in mixed species biofilm were modulated by the bacterial species and treatment compound. These results support use of natural antimicrobial compounds to target biofilms formed by pathogenic and spoilage bacteria to improve the hygiene and food safety in food processing environments.

LIST OF ABBREVIATIONS USED

ATP	Adenosine Triphosphate
BAC	Benzalkonium Chloride
B. cereus	*Bacillus cereus*
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
CTC	5-cyano-2,3-ditolyl Tetrazolium Chloride
CV	Crystal Violet
DMSO	Dimethyl Sulfoxide
E. coli	*Escherichia coli*
E. coli O157:H7	*Escherichia coli* O157:H7
EO	Essential Oil
EPS	Extracellular Polymeric Substances
HDMS	Hexamethyldisilazane
kV	kilovolt
Lm	*Listeria monocytogenes*
L. fermentum	*Lactobacillus fermentum*
L. innocua	*Listeria innocua*
L. lactis	*Lactococcus lactis*
L. monocytogenes	*Listeria monocytogenes*
L. plantarum	*Lactobacillus plantarum*
L. sakei	*Lactobacillus sakei*
MBC	Minimum Bactericidal Concentration
MBEC	Minimum Biofilm Eradication Concentration
MBIC	Minimum Biofilm Inhibitory Concentration
MIC	Minimum Inhibitory Concentration
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaClO	Sodium Hypochlorite
OD	Optical Density

Pf	*Pseudomonas fluorescens*
P. aeruginosa	*Pseudomonas aeruginosa*
P. fluorescens	*Pseudomonas fluorescens*
P. fragi	*Pseudomonas fragi*
P. putida	*Pseudomonas putida*
PAA	Peracetic Acid
RH	Relative Humidity
RTE	Ready-to-eat
SD	Standard Deviation
SEM	Scanning Electron Microscopy
SS	Stainless Steel
SW	Saline Water
Shb	*Shewanella baltica*
Sme	*Salmonella enterica* serovar Typhimurium
Sta	*Staphylococcus aureus*
S. baltica	*Shewanella baltica*
S. enterica	*Salmonella enterica*
S. proteamaculans	*Serratia proteamaculans*
S. Typhimurium	*Salmonella enterica* serovar Typhimurium
St. aureus	*Staphylococcus aureus*
St. epidermidis	*Staphyloccous epidermidis*
St. simulans	*Staphylococcus simulans*
St. xylosus	*Staphylococcua xylosus*
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
TSB-glu	Tryptic Soy Broth + 1% Glucose
µA	Microamperes
V_{acce}	Voltage of acceleration

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CHAPTER 1 INTRODUCTION

In the food industry, one of the most crucial and challenging issues is the control and prevention of the proliferation and biofilm formation of food-borne pathogens on foods and food contact surfaces. Pathogenic bacteria commonly found in food processing environments and therefore of concern for food safety in Canada include *Listeria monocytogenes, Salmonella enterica* and *Staphylococcus aureus*. These pathogens are isolated from food processing environments mostly due to improper sanitation and disinfection regimes. Spoilage bacteria such as *Shewanella baltica* and *Pseudomonas fluorescens* are also present in food processing environments and are of concern due to their ability to grow at low temperatures and form persistent biofilms (Bagge et al. 2001; Tryfinopoulou et al. 2002; Vogel et al. 2005).

L. monocytogenes is a Gram-positive, facultative anaerobic, opportunistic pathogenic bacterium and the cause of the worldwide recognized illness, Listeriosis (Carpentier and Cerf 2010; Lambertz et al. 2013; Valderrama and Cutter 2013). *L. monocytogenes* is mainly transmitted to humans through the consumption of contaminated foods and is commonly isolated from ready-to-eat (RTE) foods such as; processed meats, cheeses, fruits and vegetables since they have higher frequencies of contamination associated with increased handling of the products and are consumed without prior heat treatment (Beuchat 1996). *Listeria* cells can withstand harsh environments such as low refrigerated temperatures, high salt concentrations, desiccation and low pH enabling them to persist during exposure to common control strategies (Gandhi and Chikindas 2007; Schmid et al. 2009).

L. monocytogenes has been recognized as a significant issue in public health and continues to cause several major outbreaks. In 2008, there was a major outbreak of *L. monocytogenes* due to contaminated luncheon meats in Canada. The outbreak resulted in 22 deaths and 56 confirmed cases of Listeriosis and was traced back to a contaminated meat slicer which, due to poor sanitation and disinfection, contained surfaces covered in *L. monocytogenes* biofilms (Weatherill 2009). As recent as January 2015, there was a major outbreak of *L. monocytogenes* in caramel apples in the United States, leading to a multistate recall and 35 cases of Listeriosis, 7 reported deaths with 11 of these cases involving pregnant women with one reported case of fetal loss (CDC, 2015).

A predominant factor that leads to bacterial persistence in food processing environments is the natural adherence of bacterial cells to contact surfaces, particularly wet surfaces with accumulated food residues and high relative humidity (RH), resulting in the formation of a biofilm (Gandhi and Chikindas 2007; Richards and Melander 2009; Simoes et al. 2010; Belessi et al. 2011; Bridier et al. 2011). Once adhered, the bacterial cells embed themselves in a matrix of extracellular polymeric substances (EPS) and become an irreversibly attached biofilm. Biofilms are known to express a higher tolerance or resistance to sanitation and disinfection regimes (Simoes et al. 2010; Bridier et al. 2011). Persistence of pathogenic bacteria, such as *L. monocytogenes* and spoilage bacteria in food processing environments have been linked to biofilm EPS formation and resistance to control strategies (Carpentier and Cerf 2011).

Resistance to biocides has been linked to EPS, which depending on the species can be composed of polysaccharides, proteins, phospholipids, lipids and nucleic acids (Simoes et al. 2010; Belessi et al. 2011; Bridier et al. 2011; Nilsson et al. 2011; Hingston et al.

2013; Valderrama and Cutter 2013). The EPS matrix acts as a barrier that protects the target microorganisms by delaying the antimicrobials from reaching the bacteria by diffusion (Richards and Melander 2009; Simoes et al. 2010; Bridier et al. 2011; Myszka and Czaczyk et al. 2011). As *L. monocytogenes* is easily transferred from biofilms on processing equipment to foods coming into contact (Vogel and Truelstrup Hansen, 2011), control of *L. monocytogenes* biofilms as well as biofilms formed by other pathogenic and spoilage microorganisms becomes extremely important for improving the safety and shelf-life of foods and reducing the occurrence of foodborne illnesses.

Many disinfection strategies are based only on the removal of planktonic bacterial cells and not biofilms; however, studies have shown that biofilms require higher doses of disinfectants (Richards and Melander 2009; Simoes et al. 2010; Nilsson et al. 2011; Vazquez-Sanchez et al. 2014). The maturity of the biofilm also plays a role in resistance to disinfection strategies, as studies have shown that mature biofilms are less susceptible than early biofilms and both are present in food processing environments (Saa Ibusquiza et al. 2010; Vazquez-Sanchez et al. 2014).

There is a growing concern among consumers with regards to the use of toxic and harmful compounds as disinfectants in the food industry, thus driving the need for more natural as well as food and environmentally safe alternatives (Tassou et al. 2004; Leonard et al. 2010). Many natural alternatives such as essential oils (EO) have been studied for their antimicrobial properties (Tassou et al. 2004; Leonard et al. 2010). EO components such as thymol and carvacrol from thyme oil, oregano oil and summer savory have strong antimicrobial properties against several Gram-positive and Gram-negative bacteria in both the planktonic cell state and biofilms (Costentino et al. 1999, Burt 2004; Valero and

Giner 2006; Upadhyay et al. 2013). However, there is a lack of knowledge on the effect of EOs and their components on *L. monocytogenes* biofilms of different maturities as well as their effect on mixed biofilms.

In food processing environments, it is more likely that bacteria such as *L. monocytogenes* would grow on food processing surfaces as members of mixed species biofilms with other spoilage and pathogenic bacteria rather than as an individual biofilm (Carpentier and Chassing 2004). Previous studies have shown that *L. monocytogenes* may be both stimulated and inhibited in mixed biofilms formed with Gram-positive and Gram-negative bacteria (Carpentier and Chassing 2004; Daneshvar Alavi and Truelstrup Hansen 2013). Some food spoilage bacteria such as *S. baltica* were shown to enhance the growth rate and final population of *L. monocytogenes* in mixed biofilms while *P. fluorescens* lowered both parameters (Carpentier and Chassing 2004; Daneshvar Alavi and Truelstrup Hansen 2013). Pathogenic bacteria may also form mixed biofilms with *L. monocytogenes,* for example *S. enterica* grew to equal proportions in mixed biofilms with *L. monocytogenes* but was outcompeted when antimicrobial treatments with benzalkonium chloride (BAC), hydrogen peroxide or peracetic acid (PAA) were used (Kostaki et al. 2012). There are several studies on *L. monocytogenes* biofilm removal, but the fate of *L. monocytogenes* in mixed biofilms exposed to natural antimicrobials is not well documented.

It is clear that proper cleaning and disinfection strategies for the control of *L. monocytogenes* in food processing environments are still lacking. This creates the need for new alternatives with the ability to successfully remove biofilms regardless of the biofilm maturity or mixes of bacteria in the biofilms. Food and environmentally friendly

natural alternatives hold promise, but there is currently a lack of knowledge of their effect on pathogenic and spoilage microorganisms in single and mixed species biofilms.

1.1 Thesis Objectives

The objective of this research was to test the hypothesis that the application of natural compounds (thymol, carvacrol, trans-cinnamaldehyde, lemongrass EO) would inhibit biofilm formation by *Listeria monocytogenes* found in single and mixed species biofilms with *Pseudomonas fluorescens, Shewanella baltica*, *Salmonella enterica* serovar Typhimurium and *Staphylococcus aureus*. The effect of applying the EOs on mature single and mixed-species biofilms on the cellular respiration and EPS removal was also investigated. In addition the proportions of each bacterial species in the final population of the dual-species biofilms were determined.

CHAPTER 2 LITERATURE REVIEW

2.1 Food Processing Related Pathogenic Bacteria

Bacterial pathogens that are of concern for food safety in Canadian food processing facilities include *L. monocytogenes*, *S.* Typhimurium and *St. aureus*. *L. monocytogenes* is a Gram-positive, facultative anaerobe, opportunistic pathogen and the cause of the worldwide recognized illness, Listeriosis (Carpentier and Cerf 2010; Lambertz et al. 2013; Valderrama and Cutter 2013). It is an indigenous organism, which is naturally occurring in soil, water and vegetation and is likely transmitted to humans through the consumption of raw or contaminated RTE foods such as fruits, vegetables, cheeses and processed meats (Gandhi and Chikindas 2007; Carpenters and Cerf 2010; Valderrama and Cutter 2013). *Listeria* cells contain small cold-shock proteins that confer cold resistance and tolerance to osmotic stress, leading to an ability to grow at temperatures ranging from 1°C to 45 °C (Gandhi and Chikindas 2007; Schmid et al. 2009).

In 2008 there was a major outbreak of *L. monocytogenes* due to contaminated luncheon meats in Canada. The outbreak resulted in 22 deaths and 56 confirmed cases of Listeriosis (Weatherill 2009). Other recent Listeriosis outbreaks include cantaloupes in the USA, associated with 147 cases of Listeriosis and 33 deaths (CDC 2012), cold luncheon meats in Denmark in 2014 resulting in 20 confirmed cases and 12 deaths (Statens Serum Institut 2014) and contaminated caramel apples in the United States with a reported 35 cases and 7 deaths (CDC 2015). Listeriosis is a serious and possibly life-threatening disease that primarily affects older adults, pregnant women, children or individuals with weakened immune systems (McLauchlin et al. 2004). Symptoms of Listeriosis include fever, muscle aches, stiff neck, confusion and gastrointestinal symptoms. For pregnant

women, although they may only experience muscle aches and fatigue, the disease can cause stillbirths, newborn infections and miscarriages. For immune compromised individuals, the disease can also lead to septicemia and meningitis, and can be fatal (McLauchlin et al. 2004; Gandhi and Chikindas 2007). On average, approximately 132 cases of Liseriosis occur annually in Canada (Public Health Agency of Canada 2012).

Salmonella is a Gram-negative, rod shaped, facultative anaerobe that causes illness if ingested (Adams and Moss 2008; Trebichavsky et al. 2010). *Salmonella'*s optimum growth temperature is 37 °C; it is heat sensitive and easily destroyed by pasteurization temperatures (Adams and Moss 2008). *Salmonella* originates from the gastrointestinal tract of birds, wild animals, rodents, reptiles and insects, and is commonly transferred to humans through consumption of improperly cooked or disinfected food and water. *S. enterica* is the most common species involved in foodborne illnesses, it consists of six subspecies and over 2500 serotypes (Coburn et al. 2007). *Salmonella* serotype Typhimurium and Enteritidis are among the most common serotypes that cause infection. *Salmonella* is ranked as one of the most successful human pathogens due to its ability to move across the gut barrier to replicate within macrophages as phagosomes (Trebichavsky et al. 2010). Is it estimated that about 4 million Canadians suffer from food-related salmonellosis each year and it's the second most frequently reported food-related illness in Canada (Public Health Agency of Canada 2015).

Symptoms of a *Salmonella* infection include gastrointestinal symptoms, fever (enteric, typhoid or paratyphoid) and abdominal cramps (Coburn et al. 2007; Adams and Moss 2008; Trebichavsky et al. 2010). Symptoms usually occur between 12 and 72 hours after ingestion and last up to 7 days. Most healthy individuals recover without treatment,

but those with weakened immune systems may develop a more serious illness that must be treated or it could become fatal. *S. enterica* serovar Typhimurium affects humans; individuals with typhoid fever carry the bacterium in their bloodstream and intestinal tract (Coburn et al. 2007). Once recovered, some individuals become carriers of the bacteria and can continue to shed it through their feces. Obtaining this illness is an indication that the food or beverage consumed was handled in an unsanitary manner or improperly treated sewage contaminated the water supply. *S. enterica* is capable of forming single and mixed species biofilms on surfaces including food processing equipment, leading to its resistance and persistence in both host and non-host environments (Knowles et al. 2005; Chorianopoulos et al. 2008; Kostaki et al. 2012; Lianou et al. 2012; Zhang et al. 2014).

St. aureus is a Gram-positive coccus and a facultative anaerobe that causes foodborne infections when its enterotoxin is consumed (Adams and Moss 2008). It is commonly associated with hospital infections but is also an issue in the food processing environment due to its ability to form highly resistant single and mixed species biofilms (Lambert et al. 2001; Nostro et al. 2007, Rieu et al. 2008; Adukwu et al. 2012; Vasquez-Sanchez et al. 2014; Zhang et al. 2014). There are 27 species of *Staphylococcus* and 7 subspecies; enterotoxin production is mainly associated with *St. aureus,* it has the ability to produce seven different toxins (Adams and Moss 2008). Its optimum growth temperature is 37 °C while enterotoxin production has an optimum temperature range of 35-40°C (Adams and Moss 2008). *Staphylococcus* species originate from the skin and mucous membranes of warm-blooded animals and is found in the nose of 25% of humans (Adams and Moss 2008). Illness due to *St. aureus* toxins is characterized by the short

incubation period, typically 2-4 hours (Adams and Moss 2008). The most common method of transmission is through human carriers of the bacteria who have contact with the food. Typical symptoms include gastrointestinal symptoms and nausea with complete recovery within a couple of days. Illnesses due to *St. aureus* infections and intoxications are under-reported due to it being short-lived with relatively mild symptoms (Adams and Moss 2008).

2.1.2 Spoilage Bacteria

P. fluorescens and *S. baltica* are two Gram-negative, rod shaped motile bacteria that play an important role in food spoilage. The psychrotropic *Shewanella* genus, which includes *S. baltica*, are hydrogen sulfide producing microorganisms that are isolated from cold water environments and fish, causing concerns for spoilage at colder temperatures (Bagge et al. 2001; Tryfinopoulou et al. 2002; Vogel et al. 2005). It acts as a spoilage organism in marine fish, vacuum packed meats and chicken due to its ability to produce volatile sulfides, amines and the fish-smelling compound trimethylamine (Bagge et al. 2001; Tryfinopoulou et al. 2002). It can also produce specific signal compounds to enhance its spoilage activity (Tryfinopoulou et al. 2002). *Shewanella putrefaciens* A2, which is the same strain as the *S. baltica* A2 used in this study, has shown to be a rapid biofilm former and reached maximum number of adhered cells (from 10^2 colony forming unit (CFU)/cm^2 to 10^5 CFU/cm^2) on a stainless steel (SS) surface within 8 hours at 25°C (Bagge et al. 2001). It has also been observed to form mixed species biofilms with *P. fluorescens* (Bagge et al. 2001) and *L. monocytogenes* (Daneshvar Alavi and Truelstrup Hansen 2013).

P. fluorescens is a concern in terms of food spoilage due to its ability to survive cold temperatures and secretion of hydrolytic enzymes, such as lipases and proteases (Rajmohan et al. 2002; Dogan et al. 2003). These psychrotropic spoilage organisms are ubiquitous in nature, water and vegetation and are commonly an issue in the dairy industry since some secreted enzymes in contaminated milk can survive pasteurization and cause spoilage such as rancidity and proteolysis. Since *Pseudomonas* species are non-pathogenic, part of resident micro-flora in many food environments and fast biofilm formers, they may also have the potential to be used as beneficial microbes in the sense that they could outcompete pathogens and other spoilage bacteria (Bagge et al. 2001; Olanya et al. 2014).

2.2 Characteristics of Bacterial Biofilms

The attachment of bacterial cells to wet surfaces occurs as a natural tendency; they then multiply and embed themselves in a matrix of EPS, forming what is known as a biofilm (Richards and Melander 2009, Simoes et al. 2010, Bridier et al. 2011). Once the cells are embedded in the EPS matrix, they are known to express phenotypes that differ from their planktonic counterparts, including an increased tolerance or resistance to biocidal treatments (Simoes et al. 2010, Bridier et al. 2011). This phenomenon is mainly induced by a non-permanent physiological adaptation to life in a biofilm; such as sessile growth, nutrient stresses and repeatedly being exposed to sub-lethal concentrations of antimicrobials.

Biofilms consist of heterogeneous micro-colonies and are formed to enhance survival, especially during high stress situations, propagation to neighbouring locations and reproductive success (Richards and Melander 2009; Bridier et al. 2011; Myszka and

Czaczyk 2011; Valerrama and Cutter 2013). The stress response is one of the biofilms' most important features, and it was previously found that in *Pseudomonas aeruginosa,* the principal regulator of a general stress response was three times more strongly expressed in a 3-day biofilm than stationary planktonic cells (Xu et al. 2001). Oxidative stress responses were also shown to be induced in biofilms of *L. monocytogenes*, *P. aeruginosa* and *Escherichia coli* (Bridier et al. 2011). Studies have suggested that biofilm cells are in the stationary growth phase due to their slow growth, altered metabolic activity and expression of *rpoS*, the Gram-negative stationary-phase sigma factor that regulates stress response factors (Myszka and Czaczyk 2011).

Biofilm resistance to cleaning, sanitation and disinfection creates a major challenge for food processing facilities (Simoes et al. 2010; Nilsson et al. 2011). Many sanitation and disinfection regimes that eliminate the bacterium in a planktonic state are ineffective on biofilms (Richards and Melander 2009). Infrequently or improperly cleaned food processing environments provide favorable conditions for growth of planktonic cells and biofilms, due to moisture, nutrients and microorganisms from raw foods coming into contact with the equipment (Myszka and Czaczyk 2011). A facility must be designed with hygienic standards in mind; cracks, corners, crevices and joints are all sites that are highly susceptible to biofilm growth (Simoes et al. 2010; Carpentier and Cerf 2011; Nilsson et al. 2011). The presence of persistent strains may indicate inadequate cleaning and disinfection procedures and the need for more effective cleaning in areas of potential design flaws and bacterial harbourage sites (Wulff et al. 2006).

2.2.1 Biofilm Formation

There are many variables that are important for successful bacterial cell adherence and formation of biofilms including pH, flow velocity, temperature, nutrients, EPS, antimicrobials, cell communication and the texture of surfaces (Simoes et al. 2010; Valderrama and Cutter 2013). Bacteria communicate through quorum sensing, allowing them to alter gene expression within the population (Richards and Melander 2009). Previous studies have shown that inhibition of certain quorum sensing pathways can lead to differences in biofilm formation and structures (Richards and Melander 2009).

There are several steps that make up the biofilm developmental cycle. The planktonic cells are transported to abiotic surfaces and if able to adhere will begin the biofilm growth cycle followed by the production of cell to cell signaling molecules (Richards and Melander 2009; Simoes et al. 2010). This step is reversible initially and becomes irreversible once the EPS production commences. Early maturation of the biofilm structure is observed when it becomes three dimensional, and unique protrusions elongate from the biomass to allow for maximum nutrient, oxygen absorption and waste disposal. The last stage of the cycle is when the mature biofilm is removed by detachment, erosion or sloughing so that cells can colonize onto new surfaces and repeat the cycle. Once cells in the biofilms reach the step of irreversible attachment it is more difficult to remove the biofilm in cleaning and sanitation efforts, and would require mechanical or chemical disruption (De Oliveira et al. 2010). Simoes et al. (2009) found that biofilms occurred in layers, each layer showed different resistances to physical and chemical stresses.

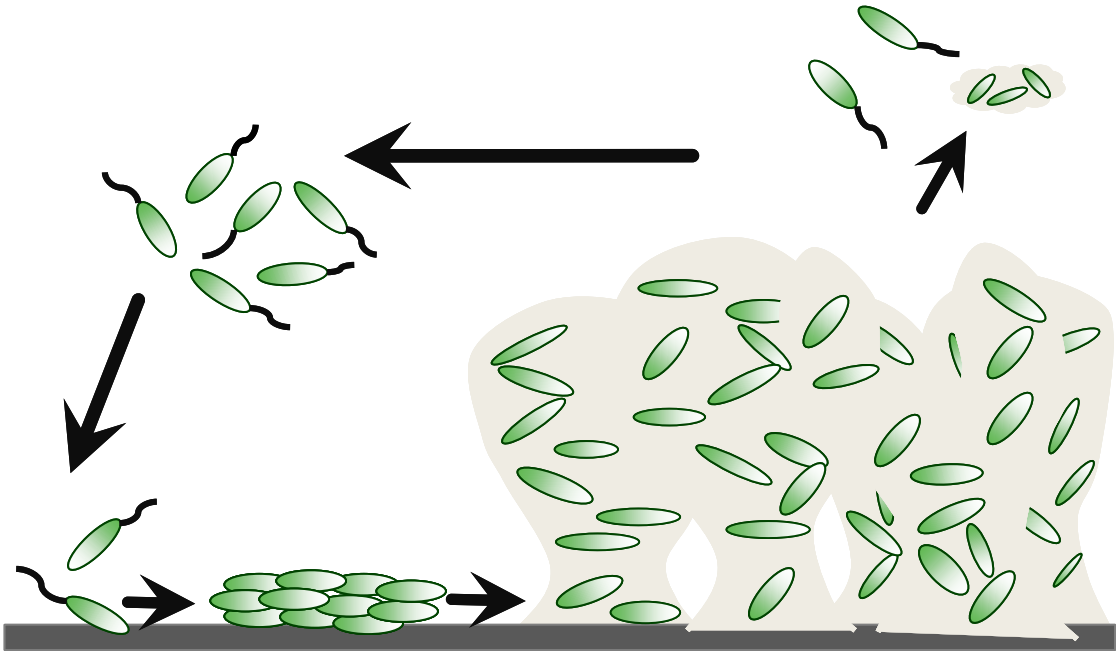


Figure 1. Biofilm formation growth cycle as described in section 2.2.1. (Used with permission Nguyen 2014)

2.2.2 Extracellular Polymeric Substances (EPS)

EPS are responsible for binding cells and other materials together as well as the adhesion to surfaces (Richards and Melander 2009; Simoes et al. 2010). Depending on the strain and growth conditions, EPS can be composed of polysaccharides, proteins, phospholipids, lipids and nucleic acids (Simoes et al. 2010, Bridier et al. 2011). The thickness of the EPS varies from 0.2 µm to 1.0 µm (Myszka and Czaczyk 2011). It has been previously found that once the cell reaches a surface, the genes encoding for flagellar proteins are repressed and genes encoding for EPS production are activated (Bridier et al. 2011).

The protective effect and persistence of a biofilm during desiccation and disinfection is dependent on the amount of time spent at the maximum cell surface density (i.e., biofilm maturity) and its ability to form the EPS (Nilsson et al. 2011; Hingston et al. 2013). The EPS matrix acts as a barrier in which the microorganism lives inside and is protected from adverse conditions such as antimicrobials; this is done by delaying the antimicrobial from reaching the target microorganism (Richards and Melander 2009; Simoes et al. 2010). An important step in the removal of biofilms is the selection of an anti-biofilm agent that will remove the targeted biofilms, preferably a product that is able to penetrate the multiple layers of EPS surrounding the microorganism (Simoes et al. 2010, Bridier et al. 2011). Many chemical and synthetic compounds such as chlorine, glutaraldehyde and sodium hypochlorite (NaClO) are based on highly reactive molecules which lose efficacy when encountering the organic matter that composes the EPS (Bridier et al. 2011). The EPS layers diffuse the antimicrobial compound resulting in lower exposure concentrations reaching the deeper regions of the biofilms and the actual target organism, causing adaptation and resistance to these concentrations (Bridier et al. 2011; Myszka and Czaczyk et al. 2011). The EPS can also trap the antimicrobial before it can cause any damage to the cells resulting in persistence of the biofilm (Richards and Melander 2009).

2.3 Biofilm Disinfection and Elimination Strategies

Prevention of the attachment and growth of pathogenic and spoilage bacteria in biofilms would ideally be more desirable then treating existing biofilms in food processing facilities. The main method of prevention and control is to clean and disinfect all surfaces before bacterial cells irreversibly attach (Simoes et al. 2010). Chemical

sanitizers and disinfectants are limited in effectiveness by the presence of organic matter, water quality and temperature (Myszka and Czaczyk 2011). Disinfectants differ from antibiotics in terms of the targeted microorganisms, disinfectants are non-specific and a broad spectrum treatment whereas antibiotics interact specifically with structures or metabolic processes in targeted cells (Bridier et al. 2011). Targeted areas in Gram-positive and Gram-negative bacteria are the cell wall, outer membrane, structural and functional proteins and genetic components.

To successfully eliminate biofilms, cleaning regimes must be able to break down, disrupt or dissolve the EPS matrix so that the disinfectants can reach the viable bacterial cell inside (Simoes et al. 2010; Bridier et al. 2011, Myszka and Czaczyk 2011). A disinfectant is only effective if the proper dose and contact time is applied to the surfaces (Simoes et al. 2010). A possible method of biofilm removal is to naturally degrade the EPS. It has been found that in instances where nutrients are depleted in the biofilm, *P. fluorescens* naturally produces enzymes to degrade the biofilm EPS to release its nutrients (Bridier et al. 2011). Processes that degrade the EPS and disperse the biofilm do not necessarily kill the bacteria but could lead to the bacteria re-depositing elsewhere. Disinfection regimes are generally very effective and eliminate the majority of surface contamination but some biofilms may persist in the environment after cleaning and disinfection causing serious public health and safety concerns and issues (Bridier et al. 2011).

2.3.1 Effect of Synthetic Disinfectants on Microorganisms

Many studies have demonstrated that biofilms are less sensitive to disinfection regimes and require higher doses than planktonic cells. Saá Ibusquiza et al. (2010)

compared the resistance of mature *L. monocytogenes* biofilms when exposed to BAC, PAA and nisin by quantifying (lethal dose 90% values (LD_{90})) the resistance of 4 and 11-day old mature biofilms. The observed resistance to BAC and nisin increased as the biofilms matured and a higher resistance to BAC and PAA was seen in biofilm cells compared to planktonic cells. Wong et al. (2010) found that *S. enterica* biofilms incubated for 3 days were less susceptible to BAC, chlorhexidine gluconate, citric acid, quaternary ammonium compounds, NaClO and ethanol, compared to planktonic *S. enterica* cells. *L. monocytogenes* cells in biofilms were found to be 1000 times more resistant to BAC than planktonic cells (Romanova et al. 2007). Since BAC is a commonly used disinfectant in the food processing industry and apparently is not effective in removing bacterial biofilms, it creates the need for new biofilm removal strategies.

The effect of the disinfection regime can also be dependent on the strain, species or maturity of the biofilm. Vazquez-Sanchez et al. (2014) examined the efficacy of BAC, NaClO and PAA on 26 *St. aureus* strains and found that disinfectant doses for food associated surfaces currently recommended by the manufacturers were lower than doses required for appropriate disinfection and complete biofilm removal. Similar to studies using *L. monocytogenes, St. aureus* biofilm cells were more resistant than planktonic cells to all treatments and mature biofilms showed higher resistance to BAC overall. It was also discovered that different strains varied in their susceptibility to individual biocides. These results show the importance of using a wide collection of strains when studying bactericidal activity and developing cleaning protocols to ensure proper dosage of disinfectants is applied. This is a significant finding in that many standardized tests

used to determine the efficacy of cleaning chemicals on food surfaces only use a few strains of each bacterium. Another consequence of the findings of Vazquez-Sanchez et al. (2014) may be the breeding of resistant strains which will survive disinfecting procedures, if low and inefficient doses are used.

In agreement with these findings, Heir et al. (2004) showed that the susceptibility of 112 human and food-related *L. monocytogenes* strains to BAC varied markedly with sensitive strains having a minimum inhibition concentration (MIC) value between 2-3 μg/ml while resistant strains exhibited MIC values between 4 and 8 μg/ml.

2.4 Effect of Natural Antimicrobials on Microorganisms

Many natural alternatives have been tested for their ability to inhibit the growth of foodborne microorganisms (Tassou et al. 2004; Leonard et al. 2010). There have been growing concerns and negative perceptions in regard to the use of synthetic and toxic biocide compounds, creating the need for more natural antimicrobial alternatives that are easily degraded in the environment and recognized as safe (Tassou et al. 2004; Leonard et al. 2010, Bridier et al. 2011). Natural plant derived compounds are generally considered to be safe for consumers and the environment as compared to synthetic, chemically based biocides (Burt 2004; Leonard et al. 2010). Among various herbs and spices showing antimicrobial activity, extracts from oregano and thyme have shown some of the best antimicrobial effects against pathogenic bacteria, and both of these spices contain carvacrol as a major component in their essential oils (EOs) (Tassou et al. 2004).

2.4.1 Antimicrobial Effect of Essential Oils

EOs are volatile, natural, phenolic compounds characterized by a strong odour and formed by aromatic plants as secondary metabolites (Bakkali et al. 2008). They

exhibit antimicrobial, antiseptic, anti-inflammatory, preservative and analgesic properties (Bakkali et al. 2008). In nature, EOs protect plants as an antibacterial, antiviral, and antifungal compound as well as an insecticide (Bakkali et al. 2008). EOs are known to elicit antimicrobial effects against a variety of bacteria such as *L. monocytogenes, S. enterica, E. coli, Bacillus cereus* and *St. aureus* at concentrations between 0.2 and 10 µL/mL (Cosentino et al. 1999; Burt 2004). Gram-negative microorganisms are slightly more resistant than Gram-positives while yeasts are the most susceptible (Cosentino et al. 1999; Burt 2004). A large variety of EO components are known for their antimicrobial properties such as; thymol, eugenol, carvacrol and cinnamaldehyde (Burt 2004). Commonly, EOs that show the strongest antimicrobial effects against food borne pathogens have high phenolic concentrations (Burt 2004). The hydrophobicity of EOs allows them to interact and separate the lipids embedded in the cell membrane, making the cell permeable and causing leakage of cell contents (Juven et al. 1994).

2.4.2 Thymol and Carvacrol

Thymol and carvacrol are major constituents of various oils such as oregano, thyme and summer savory; they are phenolic monoterpenoids and are structurally similar, with the sole difference being the position of the hydroxyl group (Figure 2) (Burt 2004; Hyldgaard et al. 2012). Their mode of action is due to their ability to position themselves in the inner and outer membranes of bacterial cells to increase cell permeability, interact with proteins and cause release of lipopolysaccharides and inorganic ions (Lambert et al. 2001; Hyldgaard et al. 2012). Previous studies investigated the effect of carvacrol on *B. cereus*, which resulted in a decrease in adenosine triphosphate (ATP) synthesis, disintegration of the phospholipid bilayer, increased permeability of the cell and

weakening of the proton motive force (Ultee et al. 2000; Ultee et al. 2002). When combined, thymol and carvacrol were shown to cause an additive effect as antimicrobials and increasing their ability to inhibit growth of spoilage and pathogenic bacteria such as *P. aeruginosa* and *St. aureus*, respectively (Lambert et al. 2001).

When added together with other antimicrobials, thymol can play a synergistic role in terms of antimicrobial activity. For example, a combination of 40 IU/mL of nisin Z, a commonly used antimicrobial, and 0.02% (w/v) of thymol resulted in a greater reduction of the growth of *L. monocytogenes* than either compound on their own (Ettayebi et al. 2000). Carvacrol has also been shown to increase in effectiveness as an antimicrobial when combined with another natural compound such as p-cymene and citral (Kisko and Roller 2005; Rattanachaikunsopon and Phumkhachorn 2010; Silva-Angulo et al. 2015).

Thymol and carvacrol have been shown to inhibit the planktonic growth of *E. coli, E. coli* O157:H7, *S. enterica, St. aureus, L. monocytogenes,* and *B. cereus* with MIC values ranging from 56.25 – 450 µL/mL (Costentino et al. 1999). Both compounds also inhibited the planktonic growth of *B. cereus* at concentrations of 0.3 and 0.15 mg/mL for thymol and carvacrol, respectively (Valero and Giner 2006). Exposure to those concentrations caused complete inhibition for 60 days at 16 °C, while lower concentrations prolonged the lag phase and reduced growth rate and final bacterial population levels. Upadhyay et al. (2013) investigated the anti-biofilm activity of trans-cinnamaldehyde, carvacrol and thymol against *L. monocytogenes* biofilms. It was observed that all compounds reduced the biofilm formation and was able to remove pre-

formed, mature biofilm formed in micro-titer plates and on SS surfaces. The concentrations that exhibited removal of pre-formed biofilm were 5.0 and 10 mM for carvacrol and trans-cinnamaldehyde and 3.3 and 5.0 mM for thymol. It was also reported that all three treatments reduced EPS production in *L. monocytogenes* biofilm on polystyrene plates after 96 hours of incubation at 37, 25 and 4 °C.

Nostro et al. (2007) studied the antimicrobial activity of thymol and carvacrol against *St. aureus* and *Staphylococcus epidermidis* strains. The study revealed that biofilm were inhibited *in vitro* at concentrations ranging from 0.031-0.125% (v/v) for all strains tested. Thymol and carvacrol also eradicated, which is defined as complete removal of biofilm growth, both *Staphyloccocus* spp. biofilms at concentrations of 0.125-0.500% (v/v). These results show that the effective concentration of treatment is dependent on the strain and that eradication of biofilm required higher concentrations than inhibition. It was also noted that the concentrations required for biofilm inhibition and eradication were up to fourfold greater than those required to inhibit planktonic cells.

Carvacrol has been demonstrated to lower viability, rapidly deplete cellular ATP and release ATP from *E. coli* and *L. monocytogenes* at a concentration of 10 mM (Gill and Holley 2006). At concentrations of 5 to 10 mM, carvacrol also inhibited the motility of *E. coli* and *L. monocytogenes*. During early biofilm development stages of *St. aureus* and *S. enterica,* carvacrol inhibited biofilm formation by 2.5 and 3 log, respectively, when cells were continuously exposed to a concentration of 1 mM of the EO (Knowles et al. 2005). Carvacrol at a higher concentration of 5 mM also effectively inhibited the formation of mature mixed species biofilm containing *St. aureus* and *S. enterica*.

2.4.3 Cinnamaldehyde

Cinnamaldehyde is a phenylpropene aldehyde (Figure 2) that is extracted from cinnamon tree bark and gives cinnamon its aroma and flavor (Muthuswamy et al. 2007; Hyldgaard et al. 2012). Aldehydes are reactive and are known to cross-link covalently with DNA and proteins to impair normal cell function, but the precise mode of action of cinnamaldehyde is not known and debatable among various studies (Gill and Holley 2004; Muthuswamy et al. 2007; Hyldgaard et al. 2012). At sub-lethal concentrations cinnamaldehyde acts as an ATPase inhibitor and at lethal concentrations it disrupts the cell membrane (Hyldgaard et al. 2012).

Gill and Holley (2004) evaluated the bactericidal effect, which in their study was defined as more than 1 log reduction in the CFUs recovered, on *L. monocytogenes* and *Lactobacillus sakei* following the exposure to cinnamaldehyde for 1 hour. A concentration of 30 mM of cinnamaldehyde was required to attain a bactericidal effect on *L. monocytogenes* while the maximum concentration of 500 mM was ineffective against *L. sakei.* When treated with a 40 mM concentration of cinnamaldehyde for 5 minutes, a significant reduction of the cellular ATP of *L. monocytogenes* was observed (Gill and Holley 2004). Cinnamaldehyde can also reduce the cell motility of *L. monocytogenes* and *E. coli* when treated at concentrations of 5 mM and 10 mM over a 10 minute period (Gill and Holley 2006). Kim et al. (2015) investigated the antimicrobial properties for cinnamaldehyde and 83 other essential oils against *P. aeruginosa*, among which only cinnamon bark oil and cinnamaldehyde significantly reduced the bacterial growth. At concentrations of 0.05% and 0.01% (v/v) cinnamaldehyde had significant inhibitory effects on *P. aeruginosa* and enterohemorrhagic *E. coli* O157:H7 biofilms, respectively. *P. aeruginosa* planktonic growth required a concentration of 0.1% (v/v) to be noticeably

inhibited, suggesting that cinnamaldehyde may have stronger anti-biofilm effects than antimicrobial effects.

Cinnamaldehyde is also an effective antimicrobial against mixed species biofilms. Zhang et al. (2014) observed the effects of cinnamaldehyde on single and mixed species biofilms of *S. enterica* serovar Enteritidis and *St. aureus*. The MIC, described as the lowest concentration to show no optical density (OD) growth after 24 hours at 37 °C, was 0.4 μg/mL for both bacteria individually. When formed in mixed species biofilms, *St. aureus* dominated the biofilm. The mixed biofilm was reduced when treated with cinnamaldehyde concentrations ranging from 0.1 μg/mL to 0.4 μg/mL.

Muthuswamy et al. (2007) found that an ethanol extract of cinnamon bark inhibited growth of *E. coli* O157:H7 and *Listeria innocua* by 94 and 87%, respectively. A cinnamaldehyde concentration of 2 mM or higher inhibited the growth of planktonic *E. coli* O157:H7 and *L. innocua*. Cinnamaldehyde has also been shown to extend shelf life of fruits and fruit juices and reduce pathogens (Mosqueda-Melgar et al. 2008; Raybaudi-Massilia et al. 2008). Cinnamon bark oil at a concentration of 0.2% (v/v) reduced the growth of *E. coli* O157:H7, *L. monocytogenes* and *S. enterica* Enteritidis by more than 5 log CFU/mL in various melon juices giving them a minimum shelf life of 91 days (Mosqueda-Melgar et al. 2008). However, these authors also mentioned one major disadvantage being that taste and odour were significantly affected, thus driving the need for further studied focused on reducing the sensory effects of the compounds. When incorporated into edible coatings for freshly cut apples, cinnamaldehyde at a concentration of 0.5% (v/v) reduced the growth of *E. coli* O157:H7 by 4 log CFU/g (Raybaudi-Massilia et al. 2008).

2.4.4 Lemongrass Essential Oil

Lemongrass EO, derived from the plant *Cymbogon citratus,* has been shown to inhibit and prevent biofilm formation by bacteria such as *St. aureus* and *L. monocytogenes* (De Oliveira et al. 2010; Aduku et al. 2012; Hyldgaard et al. 2012). The major components of lemongrass EO are geranial and neral, and minor components include myrcene, and linalool (Figure 2) (De Oliveira et al. 2010; Adukwu et al. 2012; Hyldgaard et al. 2012). The major components of this EO are classed as monoterpenes. Monoterpenes' mode of action involves toxic effects to the structure and function of the bacterial cell membrane. Due to the oil's lipophilic characteristics, it is attracted to the membrane structures and once it accumulates, the bacterial membrane will become permeable (Sikkema et al. 1995). Permeability of the membrane is associated with disintegration of the proton motive force, a decrease of ATP, pH and electric potential and a loss of ions (Bakkali et al., 2008).

Several studies have demonstrated the antimicrobial and some antibiofilm effects of lemongrass EO. Adukwu et al. (2012) found that lemongrass EO inhibited the growth of a series of *St. aureus* strains with MIC of 0.06% (v/v). A bactericidal effect was observed for all the tested strains at a concentration of 0.125% (v/v). Lemongrass EOs also prevented *St. aureus* biofilm formation at concentrations of 0.06% to 0.125% (v/v) depending on the strain but did not effectively remove already formed biofilms at the maximum concentration of 4% (v/v).

Naik et al. (2010) also tested Lemongrass EO for its antimicrobial properties with similar results. MICs and MBCs found in this study both ranged from 0.06% to 0.50%

(v/v) for *St. aureus*, *B. cereus, Bacillus subtilis*, *E. coli*, and *Klebsiella pneumonia*. It was noted that Gram-positive bacteria were more sensitive to lemongrass EO than Gram-negatives. Hammer et al. (1999) found similar values when lemongrass EO was tested against a variety of pathogenic and spoilage bacteria including; *E. coli, P. aeruginosa, S. enterica* and *St. aureus* with MICs ranging from 0.03% - 1% (v/v). Inconsistent with previous studies, Sandasi et al. (2008) found that individual components of lemongrass EO, limonene, linalool and gernayl acetate and alpha pinene at a concentration of 1 mg/mL enhanced *L. monocytogenes* biofilm formation rather than inhibit or eradicate it.

Since lemongrass EO confers pleasant odours and tastes, it could potentially be added to foods as a preservative and inhibitor of food spoilage and possible pathogens. Vazirian et al. (2012) added lemongrass EO to cream filled baked goods inoculated with various pathogens. The MIC for *E. coli, Candida albicans, B. cereus* and *S. enterica* was 0.5 µL/mL and a 99.9% reduction in bacterial growth was observed when treated with 1.0 µL/mL. Lemongrass EO has also been shown to be effective at a concentration of 0.7% (v/v) against *E. coli* O157:H7 growth when added to freshly cut apples in the form of an edible coating (Raybaudi-Massilia et al. 2008). The *E. coli* O157:H7 population was reduced by more than 4 logs CFU/g and the product's shelf life was extended by more than 30 days (Raybaudi-Massilia et al. 2008). De Oliveira et al. (2013) tested lemongrass EO on *S. enterica* Enteritidis *in vitro* and in ground beef samples. A concentration of 3.90 µL/g of the EO was required to significantly inhibit growth *in vitro* but in meat samples the same concentration had limited effect on bacterial inhibition. The EO's effectiveness was likely reduced by the components in the meat samples suggesting that food samples may require higher antimicrobial doses.

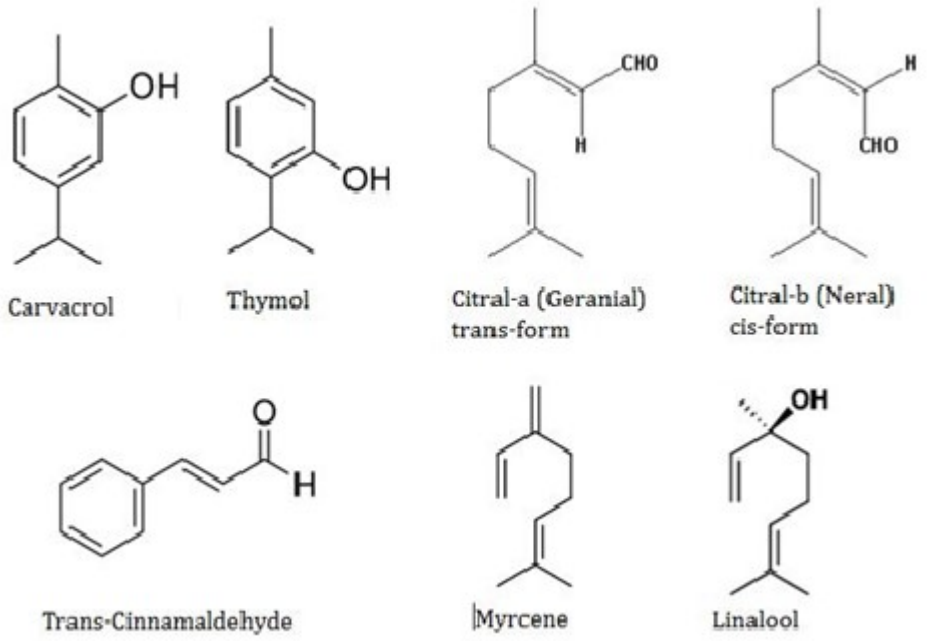


Figure 2. Chemical Structures of Essential Oil Components; Thymol, Carvacrol, Trans-Cinnamaldehyde, Citral-a (Geranial), Citral-b (Neral), Myrcene and Linalool (Adapted from: Brittanica.com, Sigmaalrich.com, and Wikipedia.org)

2.5 Mixed Species Biofilms

In food processing environments, it is more likely that bacteria, such as *L. monocytogenes*, will form biofilm together with a complex mixture of different species, than individual single species biofilms (Carpentier and Chassing 2004, Burmolle et al 2006, Bridier et al. 2011). Generally, mixed species biofilms result in a higher resistance to disinfection regimes than single species biofilms (Romanova et al. 2007; Bridier et al. 2011; Veen and Abee 2011). Possible explanations may be due to EPS formation along with chemical interactions between polymers produced by each species in the mixture, leading to a more viscous mass and less effective penetration of biocides (Bridier et al.

2011). Van der Veen and Abee (2011) studied the formation of single and mixed biofilms of *L. monocytogenes* and *Lactobacillus plantarum* and their resistance to commonly used disinfectants; BAC and PAA. They showed that single and mixed species biofilms were more resistant to BAC than planktonic cells, and mixed species biofilms were more resistant to BAC than single species biofilms.

Kostaki et al. (2012) found a mutualism between *S. enterica* and *L. monocytogenes* growing in a dual species biofilm with approximately 50% of each species present. When a disinfection assay was performed using BAC, NaClO and PAA, on both single and dual species biofilms, *L. monocytogenes* showed higher resistance. These authors concluded that although the initial growth of each species in the mixed biofilm was similar, *S. enterica* was more susceptible to the disinfection treatments causing the final biofilm community to be composed of mainly *L. monocytogenes.* Giaouris et al. (2013) formed mixed species biofilms using *L. monocytogenes* and *Pseudomonas putida* on SS coupons and treated them with BAC. Results revealed that *L. monocytogenes* increased *P. putida's* resistance to BAC while *L. monocytogenes* was mostly all killed by the disinfectant within 48 hours of incubation, leading to the biofilm population consisting of more than 90% *P. putida* cells.

Taken together, these results show that if disinfecting treatments do not successfully treat both species, the surviving species will thrive and persist in the food environment. Clearly, the effects of cleaning and disinfecting treatments depend on which species are present in mixed biofilms and their implicit properties.

2.5.1 Mixed Species Biofilms' Effect on Growth

Carpentier and Chassing (2004) studied mixed species biofilm formation of *L. monocytogenes* with 29 Gram-positive and Gram-negative bacterial strains isolated from food processing environments and discovered that some bacterial strains increased the formation of mixed biofilms with *L. monocytogenes* on SS surfaces, while others resulted in a decrease in biofilm formation or had no effect at all. Rieu et al. (2008) reported similar findings, when mixing *L. monocytogenes* EGD-e with a series of *St. aureus* strains, as growth of some *St.aureus* species increased, others decreased or remained the same in co-culture with *L. monocytogenes.*

When bacteria form mixed biofilms, it cannot be assumed that the final population is composed of equal proportions of each species. Chorianopoulos et al. (2008) formed mixed species biofilms using *L. monocytogenes, S. enterica, P. putida*, *Staphylococcus simulans* and *Lactobacillus fermentum,* and found that it was composed of 97.8% *P. putida* cells. *St. simulans* and *L. fermentum* were completely outcompeted in the biofilm mixture in the presence of the three other species while *L. monocytogenes* and *S. enterica* made up 2.2% of the bacterial population. It has also been observed in a mixed biofilm consisting of *St. aureus* and *S. enterica* that *St. aureus* dominated the mixed community by approximately 99% (Knowles et al. 2005).

Daneshvar Alavi and Truelstrup Hansen (2013) investigated biofilm formation of *L. monocytogenes* in single and dual species biofilms with *S. baltica, P. fluorescens* and *Serratia proteamaculans*. In agreement with other studies, different species had different effects on *L. monocytogenes*, where *S. baltica* significantly increased the growth rate and biofilm formation of *L. monocytogenes* whereas *P. fluorescens* and *S. proteamaculans* reduced the biofilm formation of *L. monocytogenes* (Daneshvar Alavi and Truelstrup

Hansen 2013). However, when the mixed biofilms were subjected to desiccation, survival was much higher for *L. monocytogenes* than for *S. baltica* and *P. fluorescens* thus changing the proportions of the surviving bacteria in the mixed biofilms. In accordance to these findings, Norwood and Gilmour (2001) found that *L. monocytogenes* strains *Scott A* and *FM876* demonstrated a decrease in adherence to SS in a mixed biofilm, compared to single species biofilm, due to competition when mixed with *Pseudomonas fragi* and *Staphylococcus xylosus*.

In some cases, the competition for dominance in mixed species biofilms may be beneficial in the sense that pathogenic bacteria might be outcompeted. Habimana et al. (2011) investigated the influence of *Lactococcus lactis,* a widely used dairy starter, on *L. monocytogenes* during the formation of a mixed biofilm. Results demonstrated that the resident flora inhibited the growth of *L. monocytogenes* in the biofilm. The authors speculated that this inhibitory effect could be due to the differences in generation times between the two species during early biofilm development stages.

Different species of the same bacterial genus can also affect each other during formation of mixed species biofilms. Koo et al. (2014) tested the effect of *L. innocua*, a non-pathogenic member of the *Listeria* genus, when grown together with *L. monocytogenes*. A decrease in *L. monocytogenes* attachment to SS at 24 and 72 hours was observed when *L. innocua* was present, indicating that *L. innocua* causes a decrease in *L. monocytogenes* biofilm formation. Moreover, Pan et al. (2009) formed *L. monocytogenes* mixed species cultures using strains of serotypes 1/2a and 4b and found that serotype 1/2a strains formed biofilm more efficiently and dominated in the mixed culture.

It is clear that controlling the growth of bacterial biofilms is necessary in food processing facilities to achieve, improve and maintain food safety. As previously stated, many disinfection strategies and studies are based on planktonic cell studies, omitting biofilms (Vazquez-Sanchez et al. 2014). At this time, there are many studies that have proven that cells lodged in biofilms require higher doses of disinfection compounds, which if not applied will result in the lack of biofilm removal and persistence of bacteria in food processing facilities (Simoes et al. 2010; Nilsson et al. 2011; Vazquez-Sanchez et al. 2014). Moreover, it is important to observe the effect of disinfectants on biofilms of different development stages since studies have shown that early biofilms are more susceptible than mature biofilms and both are present in processing facilities (Saá Ibusquiza et al. 2010). Since some common disinfectants have shown to only remove or kill planktonic bacteria and inhibition and removal of biofilms would require higher doses than what is recommended by the manufacturers, new strategies are needed. Plant derived natural EO compounds may hold promise; however, there is currently a lack of knowledge about the effect of EOs on *L. monocytogenes* single and mixed species biofilms.

2.6 Micro-titer Plate Screening Methods to Determine Bacterial Inhibition and Eradication

Many studies that have tested the efficacy of antimicrobial compounds against bacterial biofilm have used polystyrene micro-titer plate assays (Stepanovic et al. 2000; Pitts et al. 2003; Peeters et al. 2007; Coenye and Nelis 2010; Van der Veen and Abee 2011; Adukwu et al. 2012; Upadhyay et al. 2013; Kim et al. 2015). In this type of assay, biofilms will form on the bottom and walls of the wells of the plate (Djordjevic et al 2002; Coenye and Nelis 2010). Micro-titer plates allow for multiple organisms and/or

treatments to be tested in a single experimental run (Djordjevic et al 2002; Peeters et al. 2007; Coenye and Nelis 2010). They are easy to handle, versatile, fast and efficient, requiring only small volumes of reagents (Coenye and Nelis 2010).

Bacterial cells grown in the polystyrene plates can then be stained and enumerated by absorbance readings to allow for a more rapid screening of the antimicrobial compounds and their effect on the bacteria rather than traditional plating and enumeration methods (Pitts et al. 2003; Coenye and Nelis 2010). These methods are sensitive enough to explain and understand the concentration-response relationship and differences between species and antimicrobial agents including plant extracts (Pitts et al. 2003; Coenye and Nelis 2010). The micro-titer plate methods allows for the determination of minimal concentrations that inhibit and eradicate planktonic cells and biofilms (Pitts et al. 2003; Nostro et al. 2007; Adukwu et al. 2012; Jadhav et al. 2013; Upadhyay et al. 2013). Micro-titer plates have been shown to yield similar trends and results as traditional methods as well as other materials, confirming that it is an effective method to test differences in biofilm formation (Djordjevic et al. 2002). Staining different components in the biofilms can give information on the removal of the biofilm and the ability to disinfect it, which traditional methods lack (Pitts et al. 2003). Although staining methods can quantify biomass and viability of the cells, they cannot differentiate between species unless other methods such as fluorescent dyes are used (Hannig et al. 2010).

2.6.1 Crystal Violet Staining of Bacterial Biofilm Biomass

A common stain used when screening bacterial biofilm formation with or without antimicrobials is crystal violet (CV) (Stepanovic et al. 2000; Pitts et a. 2003; Peeters et al. 2007; O'Toole 2011; Adukwu et al. 2012; Lianou et al. 2012; Jadhav et al. 2013; Banat et

al. 2014). CV staining measures the amount of biomass or biofilm by binding to negatively charged surface molecules, but as a disadvantage, it does not measure bacterial viability (Li et al. 2003; Pitts et al. 2003; Jadhav et al. 2013). This staining method is used to determine if the antimicrobial treatment removed the biofilm biomass but not whether it killed or inhibited the bacterial cells. Stained biofilms, or their EPS and extracellular proteins, are quantified by absorbance readings of the CV stain that reacted with the biofilm in the micro-titer plate, where a greater absorbance is assumed to be proportional to the amount of biofilm biomass (Banat et al. 2014). The advantages of this method are that biofilm inhibition assays can be measured directly from the micro-titer plate rather than using plating or planktonic cell techniques.

Stepanovic et al. (2000) modified the traditional crystal violet staining procedure to improve the micro-titer plate technique for quantification of biofilm. The traditional staining method involved staining the micro-titer plate's wells and reading the OD of the dried stain. A modified procedure involved solubilizing the stain with 33% glacial acetic acid and then reading the absorbance. Results showed that the modified method was superior in its ability to quantify the biofilm biomass because it measured the sum of stain bound to the bottom, sides and walls of the wells while the standard test only measured stain attached to the bottom (Stepanovic et al. 2000). The crystal violet staining used in this present study is a modified method adapted from Nguyen et al. (2011) which incorporated the solubilisation of the stain with 33% acetic acid and the use of pin or peg lids. The pin lid is a lid which has 96 protrusions or pins on the inside and fits into a 96-well micro-titer plate bottom. After inoculating each well, biofilms are allowed to form on the pins rather than the bottom of the well. The pin lid system has

been found to provide more uniform results in assays to determine the amount of biofilm attached to a surface and reduce the difficulty in distinguishing between attached sessile cells and planktonic cells in the 96-well micro-titer plate (Junker et al. 2007). The pin lid is easily rinsed, inoculated, treated, stained, and disposed allowing for a more rapid screening assay (Junker et al. 2007).

2.6.2 Quantification of Metabolic Activity in Bacterial Biofilms

Metabolic activity stains have shown to be effective for a broad range of microorganisms (Pitts et al. 2003; Peeters et al. 2007; Adukwu er al. 2012; Jadhav et al. 2013). Commonly used metabolic activity stains involve the use of tetrazolium salts such as (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide (MTT), 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) and 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5 [(phenylamino)carbonyl]-2H-tetrazolium hydroxide (Pitts et al. 2003; Peeters et al. 2007; Adukwu er al. 2012; Jadhav et al. 2013).

MTT can be used in a simple staining assay that allows for quantification of viable or metabolically active bacteria using a micro-plate absorbance reader. The water soluble yellow MTT stain is reduced to water-insoluble purple formazan crystals by dehydrogenase enzymes in living bacterial cells. The reduced formazan crystal is quantified by measuring the absorbance of the stained micro-titer plate wells after the crystal is dissolved by an organic solvent, such as dimethyl sulfoxide (DMSO). The concentration from the absorbance reading is directly proportional to the amount of metabolically active cells in the sample (Molecular Probes Inc. 2002; Wang et al. 2010).

2.6.3 Scanning Electron Microscopy (SEM)

Microscopic techniques for the assessment of biofilm, unlike spectrophotometric techniques, can provide semi-quantitative data on the number of sessile cells as well as a detailed structural characterization for comparison purposes (Gomes et al. 2013). SEM allows for three-dimensional, micro-scale observations of biofilm morphology, structure, spatial distribution and reduction when treated, but will not provide information on the interior of the biofilm (Hannig et al. 2010; Alhede et al. 2012; Gomes et al. 2013). SEM has been a predominant choice in microscopic evaluations of biofilms because it can produce clear and detailed images of microorganisms across a wide range of magnifications (Stewart et al. 1995; Norton et al. 1998; Alhede et al. 2012). Individual cells can be distinguished when viewed under the some of the highest magnifications (Stewart et al. 1995). One of the disadvantages of SEM is the difficulty in sample preparation, and the fact that the fixation and dehydration procedures may induce shrinkage due to the biofilms' high water content or structural changes to the biofilm (Hannig et al. 2010; Alhede et al. 2012). Also, species differentiation is not possible in SEM unless morphological differences exist among the microbes in the biofilms (Hannig et al. 2010; Daneshvar Alavi and Truelstrup Hansen 2013).

Gomes et al. (2013) used SEM and could observed that bacterial adhesion varied across the wall of the micro-titer plate and higher attachment of cells occurred closer to the air-liquid interface rather than the bottom regions of the well. They were also able to observe differences among biofilm structure in both locations as well as the morphology differences for treatments with and without shaking conditions. Leonard et al. (2010) used microscopy methods to evaluate the effects of EOs on biofilm and concluded that SEM was a useful and effective tool to confirm reductions or enhancements of growth of

the biofilm upon treatment with EOs. Several other studies have used SEM as a tool in the study of the effects of various treatments on bacterial biofilms, and shown it to effectively supplement staining and other quantitative and qualitative analyses (Sandasi et al. 2008; Leonard et al. 2010; Daneshvar Alavi and Truelstrup Hansen 2013; Gomes et al. 2013; Banat et al. 2014).

SEM requires a high vacuum for evaluation and because biological samples have non-conductive properties, fixation, dehydration and coating with a conductive metal is required (Hannig et al. 2010). The biofilms are fixed with aldehydes in cacodylate buffer then dehydrated in a series of ascending concentrations of ethanol (Austin and Bergeron 1995; Hannig et al. 2010). This method allows for the water to be gradually replaced by the organic solvent (Hannig et al. 2010). The samples are then dried without destruction of the structure by critical point drying, which is achieved by gradually replacing the ethanol with hexamethyldisilazane (HDMS) (Austin and Bergeron 1995; Hannig et al. 2010). Once the samples are dried, they must be coated with a conductive metal such as gold (Austin and Bergeron 1995; Hannig et al. 2010).

The SEM uses a focus of high-energy electrons to generate a variety of signals at the surface of solid samples. These signals come from electron-sample interactions and reveal information such as sample morphology, orientation of materials and quantity of sample (Swapp 2015). Two-dimensional photo-micrographs can be produced by capturing specific surface areas of the sample at magnifications ranging from 20 x to 30,000 x and spatial resolution of up to 50-100 nm (Swapp 2015). The images are produced by the detection of secondary electrons and backscatter electrons emitted by the atoms excited by the kinetic energy of the electron beam (Swapp 2015).

CHAPTER 3 MATERIALS AND METHODS

3.1 Bacterial Strains and Culture Conditions

Three strains of *Listeria monocytogenes* were used; strain 085578, isolated from human blood and known to cause illness, strain 568, isolated from a food processing plant and CP45-1, an environmental strain isolated from the Lake Fletcher watershed, Nova Scotia, Canada, (Gilmour et al. 2010; Kalmokoff et al. 2001; Stea et al. 2015). *Shewanella baltica* (isolated from spoiled cod, Jorgensen et al. 1998), *Pseudomonas fluorescens* (American Type Culture Collection [ATCC] 13525, Manassas, VA, USA), *Salmonella enterica* serovar Typhimurium (ATCC 14028) and *Staphylococcus aureus* (ATCC 25923) were also used in this study. Each bacterial strain was maintained in Tryptic Soy Broth (TSB) (Difco, BD Canada, Oakville, ON) supplemented with 1% glucose (TSB-glu) (Fisher Scientific, Fair Lawn, NJ, USA). Stock cultures were stored at -80 °C in TSB-glu with 15% glycerol (Fisher Scientific).

3.2 Preparation of Cultures

Prior to each experiment, the bacterial cultures were removed from the -80 °C freezer and partially thawed. The strains were pre-cultured in 10 mL of TSB-glu and incubated at 15 °C for 48 hours to reach the stationary phase (Daneshvar Alavi and Truelstrup Hansen 2013). Cultures were then standardized using the following procedure. An aliquot of the culture was removed and diluted 1:10 in TSB-glu, and the absorbance was measured at 450 nm using a nanophotometer (NanoPhotometer™ P-Class, Implen Inc, Westlake Village CA, USA) to determine approximate cell concentration (CFU/mL).

For each culture, two millilitres of the pre-culture was dispensed into 15 mL sterile centrifuge tubes and centrifuged for 10 minutes at 8,965 × *g* (Universal 32 R Hettich Zentrifugen, Andreas Hettich GmbH & Co, Tuttlingen, Germany). The supernatant was discarded and based on the initial absorbance reading, the pellet was re-suspended in TSB-glu to obtain a bacterial concentration of approximately 10^9 CFU/mL and then further diluted to obtain the desired initial bacterial concentration. To ensure the standardization of cultures was yielding desired concentrations, the diluted standardized culture was spot plated on Tryptic Soy Agar (TSA) plates (Difco) and incubated at 37 °C for 24 hours for *L. monocytogenes, S.* Typhimurium and *St. aureus* followed by colony enumeration. *P. fluorescens and S. baltica* were incubated at room temperature (22 °C) for 48 hours and enumerated.

3.3 Natural Antimicrobial Compounds

The antimicrobial and anti-biofilm properties of the following four natural compounds were tested at 10 concentrations obtained by a 2-fold dilution series: thymol (≥ 99.5% pure) at 0.06, 0.12, 0.25, 0.50, 1, 2, 4, 8, 16 and 32 mM, carvacrol (99% pure) and trans-cinnamaldehyde (99% pure) at 0.045, 0.09, 0.18, 0.375, 0.75, 1.5, 3, 6, 12 and 24 mM, and lemongrass EO (natural from *Cymbopogon citratus* D.C.) at 0.03, 0.06, 0.12, 0.25, 0.50, 1, 2, 4, 8, 16 % (v/v). All four compounds were purchased from Sigma Aldrich Co. Ltd. (St. Louis, MO, USA).

3.4 Determination of Minimum Inhibitory (MIC) and Minimum Biofilm Inhibitory Concentrations (MBIC)

One hundred microliters of a standardized bacterial culture, prepared as described above, were added to wells of a sterile 96-well micro-titer plate (Costar 3370, Corning

Inc. Corning, NY, USA) at an initial concentration of 10^3 CFU/mL. This was done for all three *L. monocytogenes* strains as well as *S. baltica*, *P. fluorescens*, *S.* Typhimurium and *St. aureus*. To test the inhibitory effects of the natural compounds, each bacterium was treated with the range of concentrations listed in section 3.3 for each antimicrobial compound. Each antimicrobial treatment concentration was tested in triplicate together with a positive control containing TSB-glu and bacterial cells, a negative control containing TSB-glu and the antimicrobial and a medium only control to test sterility.

The plates were then sealed using a polyester adhesive seal (Nunc,Thermo Fisher Scientific, Rochester, NY, USA) to prevent the vapours from the essential oils affecting surrounding wells and the exterior of the plate was wrapped with parafilm (Parafilm M®, Bermis NA, Neenah WI, USA) to avoid evaporation. The plate was incubated at 15 °C in a desiccation chamber (Scienceware Desiccator Cabinet, Fisher Scientific, Ottawa, ON, Canada) with the RH adjusted to 100% by placing two petri dishes with water on the bottom shelf, for 48 hours for *P. fluorescens* and *S. baltica* and 72 hours for all other species. Incubation times were determined based on preliminary experiments, as those that resulted in consistent staining assays. Once the incubation was completed, 75 µL from each well was removed and dispensed in a new sterile 96-well micro-titer plate. The absorbance at 490 nm in the wells of the new micro-titer plate, now containing the planktonic cells, was measured using a microplate reader (ELx808™ Absorbance Microplate Reader, Biotek® Instruments Inc., Highland Park, VT, USA). The absorbance was used to calculate the percent growth inhibition caused by each treatment concentration compared to the positive and negative controls. From this the MIC was

determined as the lowest antimicrobial treatment concentration that significantly ($P \leq 0.05$) inhibited growth of planktonic cells compared to the positive control.

The MBIC was determined by using the original plate once planktonic cells were carefully removed. The metabolic activity of the bacterial biofilm remaining in the wells was determined using the MTT staining assay (Life Technologies, Eugene OR, USA) (section 3.8). The lowest concentration of the treatment compounds to significantly ($P \leq 0.05$) inhibit the metabolic activity of biofilm compared to the positive control was determined as the MBIC.

Pitts et al. (2003) showed that row H and column 1 of a 96 well micro-titer plate gave consistently lower absorbance readings than all other wells on the plate. For these reasons, the outer rows (A and H) and columns (1 and 12) of the 96 well micro-titer plates were omitted in all experiments.

3.5 Determination of the Minimum Bactericidal Concentration (MBC)

To determine the minimum bactericidal concentration (MBC), the entire content (75 μL) of wells in the MIC plates, where absorbance readings indicated inhibition, were spot plated on TSA and incubated at 37 °C for 24 hours for *L. monocytogenes* species*, S.* Typhimurium and *St. aureus* or room temperature (22 °C) for 48 hours for *P. fluorescens* and *S. baltica.* The lowest concentration which resulted in no visible bacterial growth was considered to be the MBC (Adukwu et al. 2012; Jadhav et al. 2013).

3.6 Determination of the Minimum Biofilm Eradication Concentration (MBEC)

In this assay, the antimicrobial compounds were added to pre-formed bacterial biofilms. Biofilms were pre-formed by the addition of 100 µL of a 48 hour standardized bacterial culture and 100 µL of TSB-glu to a sterile 96-well micro-titer plate. The exterior of the plate was wrapped in parafilm and incubated at 15 °C. *P. fluorescens* and *S. baltica* were incubated for 48 hours while 72 hours of incubation was required for all other species in a desiccation chamber (100% RH). For this assay, the cultures were standardized to yield an initial bacterial concentration of 10^3, 10^5 or 10^7 CFU/mL and therefore obtain biofilms of low, intermediate and high maturities (Hingston et al. 2013). Following incubation, the absorbance of each plate was measured at 490 nm and the planktonic cells were removed by gently pipetting the liquid from each well. With the exception of thymol, a two-fold concentration dilution series was made for each antimicrobial in TSB-glu followed by the addition of 100 µL of each concentration to three wells containing pre-formed biofilm. A negative control consisting of TSB-glu and the antimicrobial compound only, a positive control of TSB-glu and bacterial cells without antimicrobials and a media only control, to ensure sterility, were also plated. The micro-titer plate was then sealed as before, wrapped in parafilm and re-incubated for 48 hours at 15 °C. This treatment is intended to simulate the effect of applying the antimicrobials on surfaces in food processing plants over an idle period such as the weekend. The metabolic activity remaining in the biofilm in the wells was then measured using the MTT assay (section 3.8). The MBEC is recorded as the lowest concentration which reduced the bacterial metabolic activity by 95% ± 5% compared to the positive control (Adukwu et al. 2012; Jadhav et al. 2013).

3.7 Determination of Minimum Inhibitory Concentrations for Thymol

The preparation of the essential oil compound thymol required the compound to be dissolved in DMSO (DMSO BP231-1, Fisher Scientific). Assays using thymol were conducted at different volumes to equalize and minimize the effect of the solvent on the bacterial cultures. A 2.4 M stock solution of thymol was prepared and a two-fold dilution series using DMSO was done to obtain previously mentioned concentrations (section 3.3) when added to the plate with the bacterial culture. For the MIC, MBC and MBIC determinations, the effect of each concentration of thymol was tested on each bacterium by adding 2 µL thymol solution to 148 µL of a 10^3 CFU/mL bacterial culture into wells of a sterile 96-well micro-titer plate. The plate was then incubated for 48 or 72 hours at 15 °C depending on the bacterial culture, and the MIC, MBC, MBIC was determined as previously described above.

For the MBEC determination, biofilms were pre-formed as described above and the thymol stock solution was diluted in DMSO to obtain the concentrations previously mentioned (section 3.3). The pre-formed biofilm was treated by adding 2 µL of each thymol solution to 148 µL of TSB-glu. The plate was then re-incubated 48 hours at 15 °C and the MBEC was determined. In these cases, solvent controls were also plated consisting of the bacterial culture and DMSO (2 µL DMSO, 148 µL bacterial culture). DMSO at a concentration of 1.3% (v/v) had no inhibitory effects on the bacterial cultures.

3.8 Biofilm Metabolic Activity (MTT) Assay

This method measures the metabolic activity of viable cells in the biofilms (Pitts et al. 2003). Subsequent to appropriate incubatory periods as described above, 96-well

micro-titer plates for the MBIC and MBEC determination were stained with MTT using a modified protocol as recommended by the manufacturer (Molecular Probes Inc. 2002). The MTT stock was prepared by mixing the MTT powder with saline water (SW, 0.85% sodium chloride, Fisher Scientific) to obtain a stock solution of 12 mM. Following incubation, the spent medium was removed from the plate and replaced with 100 µL of fresh TSB-glu and 10 µL of the 12 mM MTT stock. The plate was spun down by centrifugation (20 s at 131×g) to ensure that the stain reached the bacterial biofilms at the bottom of the wells. The plate was incubated for two hours at 37 °C.

Following incubation, all medium was removed except 25 µL. Fifty microliters of DMSO was then added to each well and mixed by pipette to dissolve the precipitated formazan formed during incubation due to reduction of MTT by the dehydrogenase enzyme found in living cells (Jadhav et al. 2013). The plate was then centrifuged and re-incubated at 37 °C for 15 minutes. The contents of the wells were then mixed and the absorbance read at 570 nm. Using the following equation, the percent growth reduction of each treatment (T) compared to the positive (C) and negative (B) control was calculated. The lowest concentration of the antimicrobial compound to significantly ($P \leq 0.05$) inhibit the formation of biofilm (MBIC) and eradicate existing biofilm by 95% ± 5% reduction (MBEC) was determined.

$$\%\ Reduction\ = \frac{(C-B)-(T-B)}{(C-B)} \times 100\ \%\ \text{(Equation 1)}$$

3.9 Crystal Violet (CV) Staining

This staining method measures the amount of biofilm biomass that is present in the formed biofilms. Due to a lack of success in staining the biofilm formed on the

bottom of 96-well plates, biofilms were formed on polystyrene peg lids (Nunc, Thermo Scientific, Denmark). The 96-well flat bottom plates (Nunc) were inoculated using the same template as described above for the MTT staining. The polystyrene peg lid was then placed onto the flat bottom plates, and wrapped with parafilm to avoid evaporation. The plates were incubated at 15 °C in a desiccation chamber (100% RH) for 48 hours for *P. fluorescens* and *S. baltica* and 6 days for all other species with the exception of *St. aureus* which was not tested in this assay. Preliminary experiments showed that *St. aureus* was incapable of forming biofilm on peg lids.

For the MBIC determination of *P. fluorescens* and *S. baltica,* the plate was refreshed after 24 hours and stained after 48 hours. All other cultures were refreshed every second day and stained after 6 days. Refreshing, for the MBIC assay, consisted of transferring the peg lid to a new sterile 96-well flat bottom plate where wells had been filled with a fresh volume of TSB-glu and antimicrobials.

Refreshing for MBEC determination consisted of transferring the peg lid to a new plate with wells filled with TSB-glu. In the MBEC assay, the biofilms were pre-formed for 24 hours (*P. fluorescens* and *S. baltica*) or 6 days (*L. monocytogenes* and *S.* Typhimurium) with refreshment of the TSB-glu every two days before the addition of antimicrobial compounds. The antimicrobial compounds were then added to the wells of a new sterile flat bottom 96-well plate in desired concentrations and the peg lids containing pre-formed biofilm were transferred onto the plate and re-incubated for 48 hours at 15 °C before staining.

To stain the peg lid using crystal violet (CV, Gurr®, BDH Inc. Toronto ON, Canada), a modified protocol was followed based on the work by Nguyen et al. (2011). Once the incubation period was completed, the lid was transferred to a new 96-well flat bottom micro-titer plate containing 200 µL of SW for 10 minutes to remove any loosely attached cells. Following the rinsing step, the peg lid was transferred to another 96-well flat bottom micro-titer plate containing 200 µL of 0.1% (w/v) CV for 15 minutes. To wash excess CV stain, the peg lid was then transferred to a single well wash plate (Nunc) filled with 70 mL of distilled water for 10 minutes. This step was repeated twice. The washed peg lid was then transferred to a 96-well flat bottom micro-titer plate containing 200 µL of 33% (v/v) acetic acid (99.7% acetic acid glacial, Fisher Scientific) to solubilize the stain for 15 minutes. The absorbance of the eluted CV stain was then measured at 570 nm with a shaking step, done in the absorbance reader, and used to calculate the MBIC and MBEC using the % reduction equation (Equation 1).

3.10 Safranin Staining of Staphylococcus aureus Biofilms

To measure the biofilm biomass of *St. aureus,* which does not form biofilm on pegs, wells of a 96-well micro-titer plate were stained with safranin (Safranin O, Fisher Science Education, Hanover Park, IL, USA). The culture was incubated, standardized, diluted and dispensed in a sterile 96-well micro-titer plate using the same method and layout of antimicrobial compounds as described for the MBIC and MBEC determination using the MTT staining assay. Following incubation of 6 days at 15°C, the absorbance was read at 490 nm. The planktonic cells were then gently removed using a pipette. The wells were rinsed twice for five minutes, using 200 µL SW for each rinse. The SW was then removed by pipette and 200 µL of 0.1% (w/v) safranin was dispensed into the wells

and incubated at room temperature (22 °C) for 30 minutes. Following staining, the wells were rinsed three times with 200 µL SW. The plate was then inverted to remove any remaining liquid in the wells and air dried for one hour. The dry stained wells were then solubilized by the addition of 200 µL of 33.3% acetic acid for ten minutes. The absorbance was read at 490 nm and the MBIC and MBEC were calculated as described above (Equation 1).

3.11 MBIC of Mixed Species Biofilms

Bacterial cultures were grown and standardized as previously described. The layout of the 96-well micro-titer plate in terms of the antimicrobial compounds and controls was also the same as described above for the assay for MBIC determination for bacteria in single species biofilms.

Mixed biofilms and mixed species planktonic growth were formed by mixing 50 µL of a bacterial suspension of *L. monocytogenes 568* (10^3 CFU/mL) with 50 µL of *S. baltica*, *P. fluorescens*, or *S.* Typhimurium separately. The plates were then sealed with a polyester adhesive seal, wrapped with parafilm and incubated for 72 hours at 15 °C. Following incubation the mixed biofilms were stained using the MTT assay, and the MBIC was determined as previously described. When testing the effect of thymol, 2 µL of each thymol concentration was added together with 74 µL of each of the bacteria to a total volume of 150 µL.

3.12 MBEC of Mixed Species Biofilms

All bacterial cultures were pre-cultured, standardized and diluted as previously described for the MBEC of single species biofilms. Mixed species biofilms were pre-

formed by dispensing 50 µL of a bacterial suspension *L. monocytogenes* 568 together with 50 µL of either *S. baltica*, *P. fluorescens* or *S.* Typhimurium (all cultures standardized to an initial concentration of 10^3 CFU/mL) and 100 µL TSB-glu. The plate was incubated for 72 hours at 15 °C in a desiccation chamber with 100% RH. All combinations were done in triplicate in the 96-well micro-titer plate with the same positive and negative controls as in the single species assays. Following incubation, the planktonic cells were removed and 100 µL of antimicrobial treatments were added using the concentrations obtained by the same twofold dilution series to obtain ten different concentrations as before (section 3.3). The micro-titer plate, now containing the biofilms and the antimicrobials, was re-incubated for 48 hours at 15 °C. The plate was then stained using the MTT assay to determine the MBEC.

3.13 Enumeration of Bacteria from Mixed Species Biofilms on Selective Agars

To determine the proportion of each bacteria in the mixed bacterial biofilms, the mixtures were plated on selective agars. The selective agars used were *Listeria* selective agar base (Oxford formulation, CM0856, Oxoid, Nepean, ON, Canada) for *L. monocytogenes 568*, *Pseudomonas* agar base (CM0559, Oxoid) for *P. fluorescens*, Iron Agar (Gram et al. 1987) for *S. baltica* and Brilliant Green Agar (CM0329, Oxoid) for *S.* Typhimurium. The mixed bacterial species biofilms were formed as previously described, however, only selected concentrations of the antimicrobial compounds were plated.

For the MBIC, mixed species biofilms formed with the calculated MBIC and ½ MBIC values were plated in 96 well plates and incubated for 72 hours at 15 °C. For the

MBEC, the mixed biofilms were pre-formed (3 days, 15 °C) followed by the addition of 100 µL of the antimicrobials at the calculated MBECs and re-incubated for 48 hours at 15 °C, followed by plating and enumeration. In situations where the natural compounds were unable to eradicate the mixed biofilm (i.e., MBEC> the highest concentration), the highest tested concentration was applied. The number of each bacterium in the untreated, positive control biofilms were enumerated after 3 days incubation at 15 °C, corresponding to day 0 of the treatment period (T=0), and also after 2 additional incubation days (T=2, corresponding to the 48-hour antimicrobial treatment period) to observe the formation of mixed biofilm without treatment at two development stages.

To enumerate the biofilm communities (MBIC, MBEC), biofilm cells were harvested using a modified version of methods described by van der Veen and Abee (2011) and Romanova et al. (2007). Briefly, the media was removed by pipette and wells were rinsed with 100 µL SW to remove loosely attached cells. Biofilm cells were re-suspended in 100 µL of SW by pipetting rigorously approximately 6 times and scraping the bottom and sides of the well with the tip of the pipette. The contents of the well (100 µL) were then diluted appropriately for enumeration followed by spot plating of 100 µL on selective agars. Positive controls (untreated biofilms) inoculated with 50 µL of each bacterial species and 100 µL of TSB-glu were also plated on selective agars. For all plating assays, Oxford Agar and Brilliant Green Agar were incubated for 24 hours at 37 °C while *Pseudomonas* base Agar and Iron agar plates were incubated at room temperature (22 °C) for 48 hours prior to colony enumeration. The enumerated colonies were converted to log CFU/cm^2 for each of the replicate biofilm samples, averaged and

expressed as a percentage of the total biofilm population for each species in the mixed biofilm.

3.14 Scanning Electron Microscopy

To observe the effect of the antimicrobials on biofilm formation by SEM, single and mixed species biofilm were prepared by separate methodologies. Selected single species biofilms were formed on polystyrene peg lids for SEM observations. For treated biofilms, the MBICs as determined by MTT staining assays (3 day incubation at 15 °C) were chosen, in the case of *L. monocytogenes 568* for all four antimicrobial compounds while *P. fluorescens*, *S. baltica* and *S.* Typhimurium biofilms treated with MBIC levels of thymol and trans-cinnamaldehyde were selected. *L. monocytogenes 568* biofilms treated with the CV-based MBICs and MBECs following a 6 day incubation period and treatment with all four antimicrobial compounds were also observed.

The SEM fixation protocol used to prepare the samples was based on a modified version of a protocol previously described by Austin and Bergeron (1995). Following appropriate incubation periods, the peg lids were transferred to a new 96-well flat bottom plate containing 200 µL of 0.1 M sodium cacodylate trihydrate (Electron Microscopy Science (EMS), Cedarlane, Burlington, ON, Canada) solution in 2% (w/v) glutaraldehyde (50% w/w, Fisher Scientific) for two hours. Following the two hour immersion the peg lids were rinsed three times for 10 minutes each by transferring the lid into new 96-well flat bottom plates containing 200 µL of 0.1 M cacodylate buffer supplemented with 3% (w/v) glucose then transferred to a new 96-well bottom plate containing 200 µL of 1% osmium tetraoxide solution (4% aqueous solution , EMS) in 0.1 M cacodylate buffer for four hours.

The peg lid was then rinsed in 0.1 M cacodylate buffer three times for ten minutes each followed by a dehydration process using an ascending ethanol (Fisher Scientific) gradient series (35, 50, 70, 90 and 100%) for 15 minutes each, except the 100% ethanol step was done twice. The peg lid was then dried in an HDMS/ethanol (hexamenthyldisilazane, EMS) mixture series (27:75, 50:50, 75:25, 100:0) for 15 minutes each except the 100% HDMS was done twice. The fixed, dehydrated and dried peg lid was then air dried in a fume hood for 2 hours. Once fully dried, the pegs were cut off of the lid by flaming tweezers using a Bunsen burner and pinching off the pegs by melting the plastic. The pegs were then mounted on aluminum mounts (EMS) using carbon adhesive tabs (9 mm diameter, EMS) and sputter coated (Polaron-SC7620 mini sputter coater, Quorum Technologies Ltd. Canada) with Au/Pd nanoparticles (SC502-314B gold/palladium sputter target, 0.1 mm thick, Quorum Technologies Ltd. Canada). The mounts were contained in universal mount holders (EMS) in dry conditions (3-4% RH) until observed by the SEM.

The mixed species biofilms were formed as previously described for 3 days at 15°C in the absence (control) or presence of the antimicrobials at the suitable MBICs or pre-formed for 3 days at 15 °C followed by the addition of antimicrobials at the indicated MBECs and re-incubated for the 2-day post-treatment. The mixed species biofilms were formed on the bottom of polystyrene 96-well micro-titer plates following the same protocol as before (section 3.11 – 3.12). The fixation protocol remained the same, however instead of transferring peg lids to new 96-well plates filled of each fixation and dehydration solution, each solution was pipetted at the same volumes into the 96-well plate with the mixed species biofilm for the appropriate incubation period followed by

the removal by pipette to allow for rinsing or application of the next solution in the fixation protocol. The plates containing the fixed mixed biofilm were air dried for 2 hours and then the bottoms of the 96-well plate were cut out by heating a knife under the flame of a Bunsen burner. The cut 96-well plate bottom pieces containing the fixed mixed biofilm were mounted on the same aluminum stubs by tweezers and held in place by the same carbon adhesive tabs and sputter coated as described above.

The aluminum mounts containing the fixed and coated pegs or 96-well plate bottom wells pieces were placed onto the specimen holder and placed into the SEM chamber (Hitachi S-4700 FE-SEM). The biofilms were observed with the SEM using operational conditions of 10 kilovolts (kV) of acceleration voltage (V_{acce}), 14-16 microamps (μA) of emission current, 10-12 mm working distance and the analysis lens mode. Micrographs were captured at three magnifications (× 1,000, 5,000, and 10,000).

3.15 Statistical Analysis

For comparison of treatments to their corresponding positive control, the Student's t test was used to determine significant inhibition effects. Differences were considered significant when the P-value was ≤ 0.05. All analysis were computed using Microsoft Excel 2013. To compare the effect of the natural antimicrobial compounds on planktonic cells across bacterial species non-parametric Kruskal-Wallis tests were done using the calculator created by R. Lowry at Vassar College, Poughkeepsie, NY, USA (http://vassarstats.net/). Similarly, the non-parametric Friedman ranking test was used to compare the overall efficiency of the natural compounds carvacrol, trans-cinnamaldehyde and thymol for all inhibition (MIC, MTT-MBIC, CV-MBIC) and eradication (MBC, MBEC) assays on all bacteria. Lemongrass EO was omitted from this test due to

differences in concentration units (mg/mL vs. % v/v). Other non-parametric statistical tests were carried out but were not significant.

CHAPTER 4 RESULTS

4.1 The Effect of the Natural Compounds on Bacterial Planktonic Growth (MIC and MBC)

All four natural compounds were able to significantly ($P < 0.05$) inhibit and eradicate the growth of planktonic cells for all the tested bacterial species (Figure 3 A-D). The effect of these natural compounds on cell growth in 96-well micro-titer plates was dose dependent. The MICs and MBCs varied among the bacterial species and natural compounds (Figure 3 A-D). Figure 3 A-D illustrates that in all cases, the lowest dose required to attain a bactericidal effect (MBC) was higher or equal to the natural antimicrobial concentrations recorded for the inhibitory effect (MIC).

All bacterial species were inhibited by thymol at relatively low concentrations in the tested dilution series. The MICs ranged from 1 to 2 mM with the exception of *S. baltica* which was very susceptible to thymol with a MIC of 0.12 mM (Figure 3-A). The MBCs obtained were individually higher for each bacterial species with concentrations ranging from 1 to 8 mM with *L. monocytogenes 085578* as the most resistant (Figure 3-A).

Carvacrol inhibited and eradicated ($P < 0.05$) planktonic growth of all tested species (Figure 3-B) but compared to thymol and trans-cinnamaldehyde it required higher concentrations to be an effective antimicrobial (Figure 3 A-C). Similarly to thymol, *S. baltica* showed great susceptibility to carvacrol exhibiting the lowest MICs and MBCs of 0.375 and 1.50 mM, respectively (Figure 3-B). The remaining bacterial species showed a concentration range of 1.50 to 6 mM for the MICs with *L. monocytogenes 568* being the

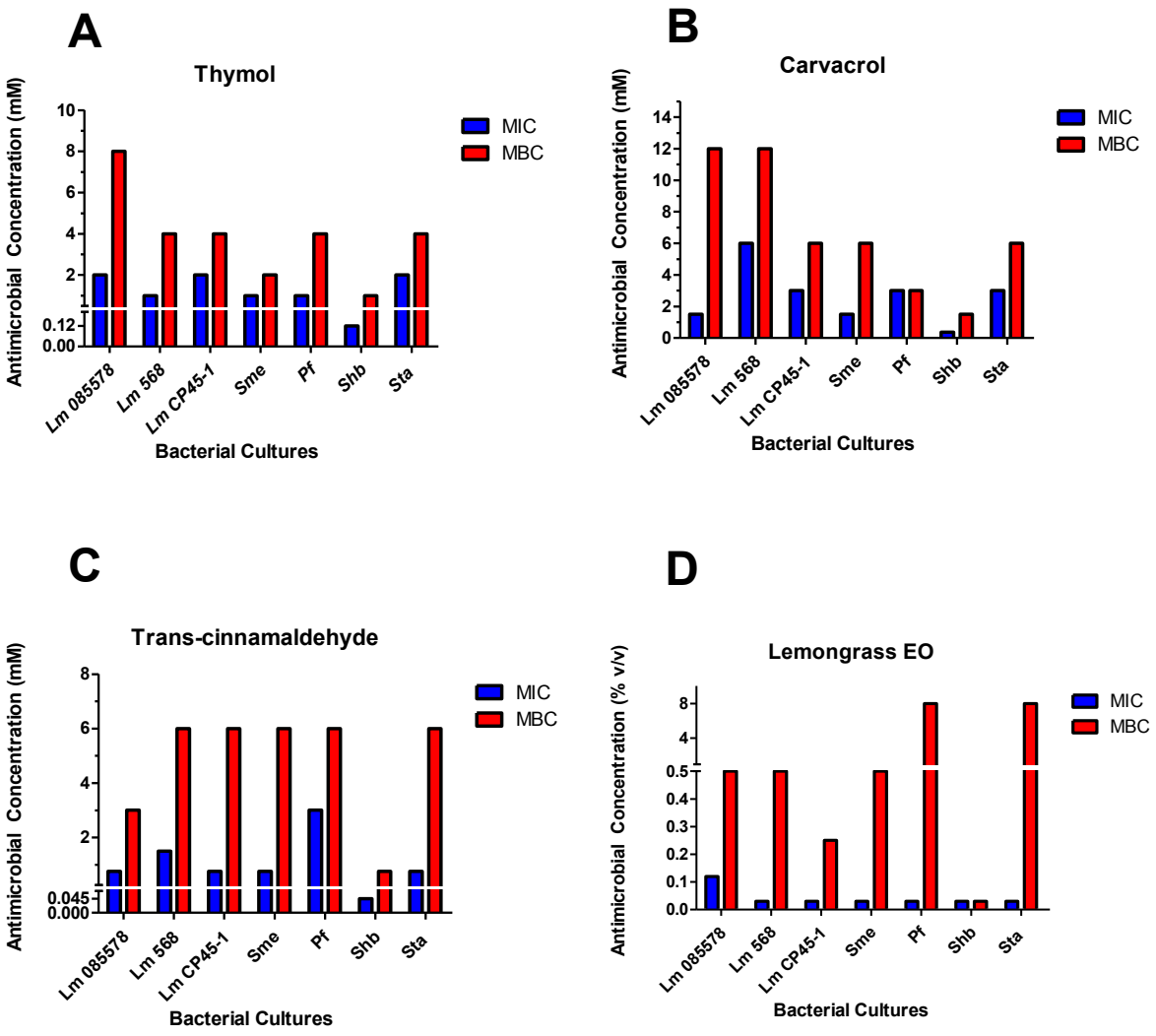


Figure 3. The minimum inhibition concentration (MIC) and minimum bactericidal concentration (MBC) for *Listeria monocytogenes* (*Lm 085578, 568, CP45-1*), *Salmonella enterica* Typhimurium (*Sme*), *Pseudomonas fluorescens (Pf), Shewanella baltica (Shb)* and *Staphyloccocus aureus (Sta)* when treated with thymol (A), carvacrol (B), trans-cinnamaldehyde (C) and lemongrass EO (D) then incubated for 48 (*Pf* and *Shb*) or 72 (*Lm, Sme* and *Sta*) hours at 15 °C.

most resistant (Figure 3-B). Planktonic cultures of *L. monocytogenes 085578* and *L. monocytogenes 568* were the most resistant to the bactericidal effects of carvacrol with MBCs of 12 mM (Figure 3-B). The MBCs for the remaining bacteria ranged from 3 to 6 mM (Figure 3-B).

As an inhibitor of planktonic bacterial growth, trans-cinnamaldehyde was one of the most effective antimicrobials, where the MICs obtained in this assay were all below 1.50 mM with the exception of *P. fluorescens*, which showed increased resistance to trans-cinnamaldehyde with a MIC of 3 mM (Figure 3-C). However, to elicit a bactericidal effect much higher concentrations were required. Five of the bacterial species had MBCs of 6 mM with the exceptions of *L. monocytogenes 085578* (3 mM) and *S. baltica.* As with thymol and carvacrol, *S. baltica* required the lowest concentrations compared to the other species of 0.045 mM and 0.75 mM ($P < 0.05$) for MIC and MBC, respectively (Figure 3-C).

Lemongrass EO significantly ($P < 0.05$) inhibited planktonic growth. With the exception of *L. monocytogenes 085578*, the MIC of lemongrass EO for all other bacteria was the lowest tested concentration of 0.03% (v/v) (Figure 3-D). For *L. monocytogenes 085578* the MIC was higher at 0.12% (v/v), which is still one of the lowest tested concentrations (Figure 3-D). For the MBCs, with the exception of *S. baltica*, much higher concentrations were required, expressing the most change between MIC and MBC compared to the other natural compounds (Figure 3-D). MBCs ranged from 0.25% to 8% (v/v); *P. fluorescens* and *St. aureus* required the second highest tested concentration of 8% (v/v) to kill the planktonic cells while their MICs were among the lowest concentration of lemongrass tested (Figure 3-D). *S. baltica* was both inhibited (MIC) and

killed (MBC) by the lowest tested concentration of lemongrass EO (0.03% v/v) (Figure 3-B). When the effect of the natural compounds on planktonic cells was compared, it was revealed that *S. baltica* is significantly ($P < 0.05$) more sensitive, followed by *S.* Typhimurium and then by *L. monocytogenes 568, P. fluorescens*, and *St. aureus*.

It is interesting to note that the susceptibility of the three *L. monocytogenes* strains that were used in this study including a clinical outbreak strain *L. monocytogenes 085578*, a food processing strain *L. monocytogenes 568* and an environmental strain *L. monocytogenes CP45-1*, varied considerably in the required antimicrobial dosage to be inhibited and/or killed. Mostly these differences were represented by a dilution factor and none of the strains could be shown to be significantly ($P \leq 0.05$) different. But these results suggested that the required concentration to inhibit and eradicate planktonic bacteria is not only species dependent but also strain dependent.

4.2 The Effect of the Natural Compounds on the Inhibition of Biofilm Formation (MBIC)

Thymol, carvacrol, trans-cinnamaldehyde and lemongrass EO all proved to be dose dependently successful in their ability to significantly ($P < 0.05$) inhibit biofilm formation of *L. monocytogenes*, *S.* Typhimurium, *P. fluorescens*, *S. baltica* and *St. aureus* in the 96-well micro-titer plate assays (Figure 4 A-D). Generally, a higher or equal antimicrobial concentration was required to inhibit the metabolic activity, measured using the MTT stain, of the biofilm than to inhibit the formation of biofilm biomass, here defined as CV (safranin for *St. aureus*) stainable EPS and cellular material irreversibly attached to the well in the micro-titer plate, in all cases except for *St. aureus* treated with

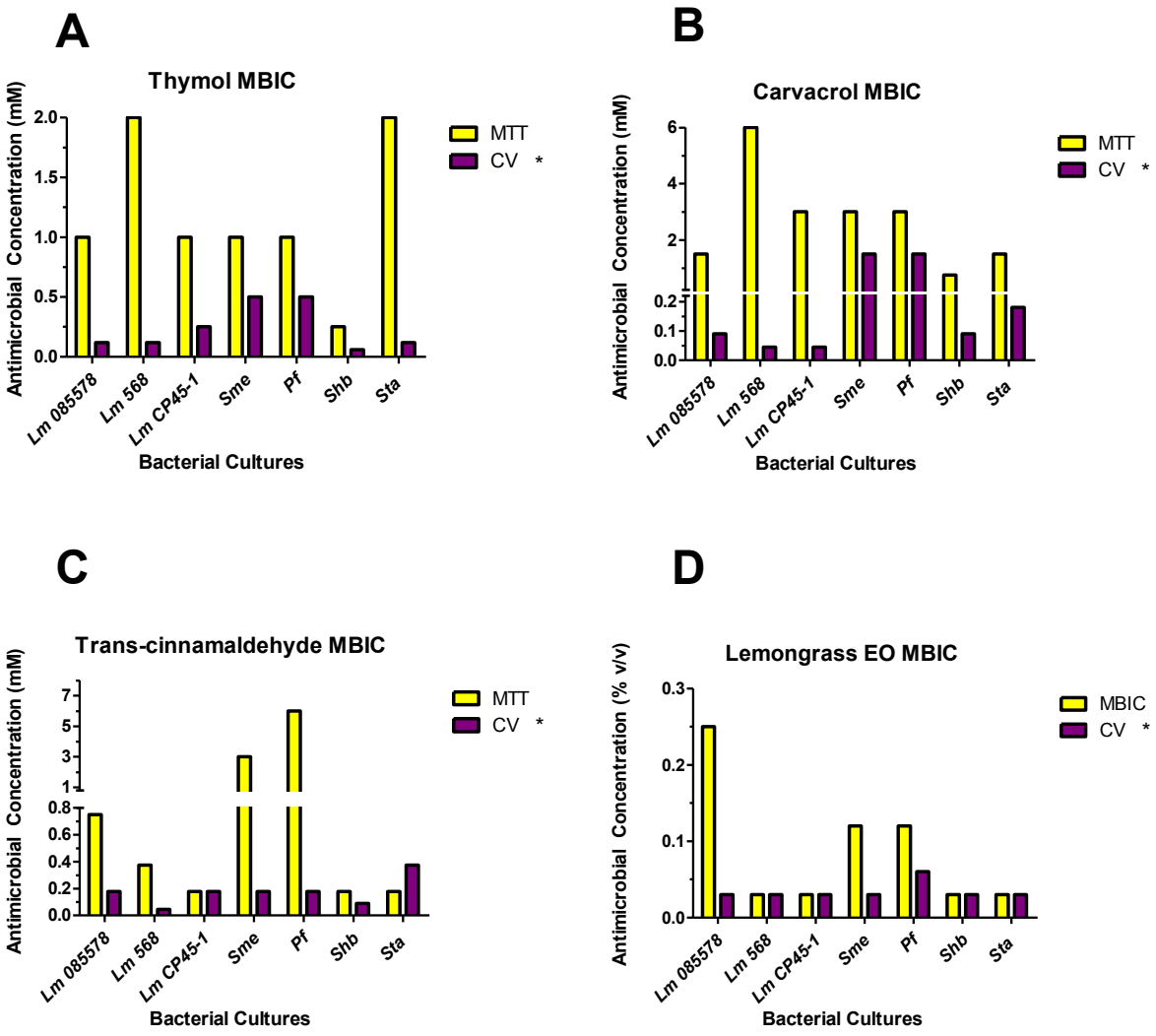


Figure 4. Comparison of the metabolic (MTT staining) and biomass (CV or safranin* staining) minimum biofilm inhibition concentrations (MBIC) for *Listeria monocytogenes (Lm, 085578, 568, CP45-1), Salmonella enterica* Typhimurium (*Sme*), *Pseudomonas fluorescens (Pf), Shewanella baltica (Shb)* and *Staphyloccocus aureus (Sta)* treated with thymol (A), carvacrol (B), trans-cinnamaldehyde (C) and lemongrass EO (D) during biofilm formation at 15 °C for 48 hours (*Pf* and *Shb*) and 3-6 days (*Lm, Sme* and *Sta*). * *St. aureus* was stained with safranin instead of CV in the biomass MBIC assay.

trans-cinnamaldehyde (Figure 4 A-D). MBICs varied greatly among bacteria and natural compounds (Figure 4 A-D).

For thymol treated biofilm, two of the Gram-positives, *L. monocytogenes 568* and *St. aureus*, showed the most resistance to the inhibition of metabolic activity with MBICs of 2 mM and the largest difference relative to the biomass formation MBICs of 0.12 mM (Figure 4-A). The metabolic activity of *L. monocytogenes 085578, L. monocytogenes CP45-1, S.* Typhimurium and *P. fluorescens* biofilm became significantly ($P < 0.05$) inhibited at the same thymol concentration (1.0 mM) (Figure 4-A). For the inhibition of biomass, two Gram-negatives; *S.* Typhimurium and *P. fluorescens* were the most resistant with MBICs of 0.50 mM compared to Gram-positives with MBICs ranging from 0.12 to 0.25 mM (Figure 4-A). *S. baltica* continued the trend of being the most susceptible to thymol also during biofilm formation with MBICs of 0.25 mM and 0.06 mM for the MTT and CV staining assays, respectively (Figure 4-A).

L. monocytogenes 568 was the most resistant bacteria when treated with carvacrol with an MBIC of 6 mM, for inhibition of metabolic activity, but interestingly its biomass was inhibited significantly ($P < 0.05$) by the lowest tested concentration of 0.045 mM (Figure 4-B). *S. baltica* was the most susceptible to carvacrol with a metabolic activity MBIC of 0.75 mM (Figure 4-B). The metabolic activity of the other tested species was inhibited at carvacrol concentrations ranging from 1.5 to 3 mM ($P < 0.05$) (Figure 4-B). In general, the carvacrol concentration required for inhibition of biomass formation (MBICs) was lower for the Gram-positive bacteria than for the Gram-negatives with the exception of *S. baltica* (MBIC of 0.09 mM). Similarly to when treated with thymol, biomass formation by *S.* Typhimurium and *P. fluorescens* were the least affected by

carvacrol and exhibited MBICs of 1.50 mM for both and the smallest difference in MBICs between the metabolic and biomass assays (Figure 4-B). The Gram-positives had biomass forming MBICs ranging from 0.045 to 0.18 mM, which represented the three lowest concentrations tested, indicating that in the presence of carvacrol Gram-positives were more susceptible to the inhibition of biomass formation than Gram-negative bacteria (Figure 4-B).

Trans-cinnamaldehyde inhibited the metabolic activity of the forming biofilm at lower concentrations compared to thymol and carvacrol for all bacteria with the exception of *P. fluorescens* which showed greater resistance to trans-cinnamaldehyde than any other bacteria with a metabolic MBIC of 6 mM while the second-most resistant bacterium, *S.* Typhimurium, exhibited an MBIC of 3 mM (Figure 4-C). The other bacteria were all effectively inhibited, in terms of metabolic activity, at concentrations ranging from 0.18 to 0.75 mM (Figure 4-C). The inhibition of biomass and EPS production occurred at the three lowest tested concentrations ranging from 0.045 to 0.18 mM ($P<0.05$) with the exception of *St. aureus*. *St. aureus* had an atypical result, where the biomass MBIC from the safranin staining assay was higher at 0.375 mM than the metabolic MBIC from the MTT staining (Figure 4-C).

Lemongrass EO greatly succeeded as an effective antimicrobial against formation of bacterial biofilm (Figure 4-D). The metabolic activity in biofilms was significantly ($P < 0.05$) repressed at the lowest tested concentration of 0.03% (v/v) for *L. monocytogenes 568, L. monocytogenes CP45-1, S. baltica* and *St. aureus* (Figure 4-D). *S.* Typhimurium and *P. fluorescens* once again showed higher resistance, with an MBIC for metabolic activity of 0.12% (v/v) (Figure 4-D). *L. monocytogenes 085578* was the most resistant to

lemongrass EO with an MBIC for metabolic activity of 0.25% (v/v) (Figure 4-D). The production of EPS/biomass was significantly ($P < 0.05$) inhibited at the lowest tested concentration of 0.03% (v/v) for all bacteria except *P. fluorescens* which required one dilution factor higher of 0.06% (v/v) (Figure 4-D). With the exception of *L. monocytogenes 085578*, lemongrass EO showed the smallest or no differences between the metabolic and biomass MBICs as compared to the other antimicrobial compounds (Figure 4-D).

Again it was noted that the inhibition of biofilm biomass formation and metabolic activity caused by the four natural compound varied among the three *L. monocytogenes* species *(085578, 568, CP45-1)* suggesting strain variations in susceptibility (Figure 4 A-D).

4.3 The Effect of the Natural Compounds on the Eradication and Removal of Pre-formed Biofilms (MBEC)

The ability of thymol, carvacrol, trans-cinnamaldehyde and lemongrass EO to inhibit or reduce the metabolic activity by >95% ± 5% in pre-formed or established bacterial biofilm of different maturities was determined as their metabolic MBECs. Table 1 clearly shows that in general, a higher or equal antimicrobial concentration is required to eradicate metabolic activity within the biofilm as it matures. For each natural compound, there were a few exceptions where it was observed that the most mature biofilm (highest initial bacterial concentration of 10^7 CFU/mL) had lower MBECs than the biofilm of intermediary maturity (10^5 CFU/mL initial bacterial concentration) (Table 1).

Table 1. Minimum biofilm eradication concentrations (MBEC) of thymol, carvacrol, trans-cinnamaldehyde and lemongrass EO for eradication of the metabolic activity (MTT) of *Listeria monocytogenes (Lm 085578, 568, CP45-1)*, *Salmonella enterica* Typhimurium (*Sme*), *Pseudomonas flurorescens (Pf), Shewanella baltica* (*Shb*) and *Staphyloccocus aureus* (*Sta*) in biofilms of three maturities; low (10^3 CFU/mL initial concentration), intermediate (10^5 CFU/mL initial concentration) and high (10^7 CFU/mL initial concentration). Values with > indicate that the antimicrobial compound was unable to eradicate the pre-formed biofilm by 95% ± 5% at the highest tested concentration.

Bacterial Species	**Thymol (mM)**			**Carvacrol (mM)**		
	Low	Intermediate	High	Low	Intermediate	High
Lm 085578	2.00	4.00	2.00	1.50	6.00	6.00
Lm 568	2.00	4.00	2.00	6.00	6.00	6.00
Lm CP45-1	2.00	4.00	4.00	3.00	6.00	6.00
Sme	2.00	2.00	2.00	3.00	3.00	1.50
Pf	8.00	8.00	>32.00	3.00	3.00	>24.00
Shb	8.00	>32.00	>32.00	12.00	>24.00	>24.00
Sta	4.00	4.00	4.00	3.00	6.00	3.00
	Trans-cinnamaldehyde (mM)			**Lemongrass EO (% v/v)**		
	Low	Intermediate	High	Low	Intermediate	High
Lm 085578	24.00	>24.00	>24.00	1.00	>16.00	>16.00
Lm 568	24.00	>24.00	>24.00	1.00	>16.00	4.00
Lm CP45-1	12.00	>24.00	>24.00	>16.00	>16.00	>16.00
Sme	12.00	12.00	6.00	0.25	0.50	0.50
Pf	6.00	6.00	3.00	8.00	8.00	>16.00
Shb	>24.00	>24.00	>24.00	0.12	>16.00	>16.00
Sta	6.00	>24.00	>24.00	>16.00	>16.00	>16.00

Thymol and carvacrol were the most effective antimicrobials as these compounds consistently were able to eliminate the metabolic activity in the least mature pre-formed biofilms (low initial concentration of 10^3 CFU/mL) for all bacteria tested as well as the more mature biofilms formed by several of the bacteria (Table 1). Interestingly, *S. baltica* exhibited the greatest resistance to eradication of metabolic activity in the pre-formed biofilms by all four compounds while opposite results were seen for *S.* Typhimurium and *P. fluorescens* biofilms (Table 1). *S.* Typhimurium was the only bacteria where all four compounds reduced the metabolic activity in its biofilms of all development stages (Table 1).

The ability of thymol to reduce the metabolic activity in pre-formed biofilms varied among the bacteria (Table 1). For *L. monocytogenes 085578* and *L. monocytogenes 568* treated with thymol, the intermediate biofilm (10^5 CFU/mL initial concentration) had a higher by one-dilution MBEC than the low and high maturity biofilms (10^3 and 10^7 CFU/mL initial concentrations) (Table 1). In contrast, *L. monocytogenes CP45-1* increased in resistance as its biofilms matured (Table 1). For *P. fluorescens* and *S. baltica*, thymol was able to eliminate metabolic activity in low maturity biofilms (10^3 CFU/mL) for both and in intermediary biofilms (10^5 CFU/mL) for *P. fluorescens,* but had no effect on sessile bacteria in the high maturity biofilms (10^7 CFU/mL) as well as on the intermediary biofilm (10^5 CFU/mL) for *S. baltica* (Table 1). Metabolic activities in *St. aureus* and *S.* Typhimurium biofilms were, regardless of the maturity of the biofilm, eradicated at the same concentration of thymol (Table 1).

Similar trends were observed when pre-formed biofilm of all species tested were treated with carvacrol (Table 1). *S.* Typhimurium and *St. aureus* required higher

carvacrol concentrations at the intermediary biofilm stage (10^5 CFU/mL initial concentration) than for the more mature biofilm (10^7 CFU/mL) (Table 1). Metabolic activity of the *L. monocytogenes* strains was effectively controlled at all biofilm maturities, with two strains requiring higher concentrations as they matured (*L. monocytogenes 085578, L. monocytogenes CP45-1)* while one was consistently inhibited by the same concentration of carvacrol (*L. monocytogenes 568*) (Table 1). Similar to thymol, carvacrol was unable to effectively eradicate *P. fluorescens* and *S. baltica* in the high maturity established biofilm (initial bacterial concentration of 10^7 CFU/mL) as well as the intermediate biofilm (10^5 CFU/mL) made by *S. baltica* (Table 1).

Trans-cinnamaldehyde was not as effective as thymol and carvacrol in the eradication of the metabolic activity in the pre-formed biofilms. At the lowest biofilm maturity (10^3 CFU/mL), MBECs ranged from 6, 12 to 24 mM, which represented the three highest tested concentrations. Moreover, the highest tested concentration (24 mM) was unable to eliminate metabolic activity in biofilms made by *S. baltica* (Table 1). For the intermediary and highest biofilm maturities, only *S.* Typhimurium and *P. fluorescens* were effectively eradicated and both required lower MBECs at the most mature biofilm stage (Table 1). Biofilms of intermediary and high maturities of all other bacteria became resistant to even the highest tested concentrations of trans-cinnamaldehyde (24 mM), meaning that the metabolic activity could not be reduced by 95% ± 5% (Table 1).

The effect of lemongrass EO varied among the bacterial species. Biofilms made by *L. monocytogenes CP45-1* and *St. aureus* were, regardless of their maturity, not affected by lemongrass (Table 1). Lemongrass EO reduced the metabolic activity in low maturity biofilms (10^3 CFU/mL) made by *L. monocytogenes 085578, L. monocytogenes*

568, S. Typhimurium*, S. baltica* and *P. fluorescens* (Table 1). *S.* Typhimurium was the only species to be eradicated regardless of the biofilm maturity, suggesting that its biofilm cells were more susceptible to lemongrass EO compared to all other bacteria (Table 1). *L. monocytogenes 568* was effectively eradicated in young and older biofilms but not in intermediary biofilms (Table 1).

The pre-formed biofilms were also stained with CV (safranin for *St. aureus*) to measure the change in biofilm biomass caused by addition of the antimicrobial compounds to biofilms of the three different maturities. Results showed that biomass removal only occurred for Gram negative bacteria (data not shown). For *S. baltica*, MBECs obtained in the biomass removal assays for all four antimicrobial compounds ranged from 0.75 to 24 mM for thymol, carvacrol and trans-cinnamaldehyde and 0.03% (v/v) for lemongrass EO in the case of the least mature biofilms (10^3 CFU/mL) and the intermediary biofilms (10^5 CFU/mL) but none of the compounds were able to reduce the biomass of *S. baltica* lodged in the most mature biofilms (10^7 CFU/mL). MBECs for the removal of *P. fluorescens* biomass in biofilms of all maturities were obtained for carvacrol and trans-cinnamaldehyde only and ranged from 3 to 6 mM with increasing concentrations as the biofilm matured. Similarly, *S.* Typhimurium pre-formed biofilm of all maturities were eradicated by thymol and carvacrol at concentrations ranging from 0.375 to 6 mM also with increasing concentrations as the biofilm matured. All biofilms, regardless of the maturity, made by the Gram positive bacteria were not affected in terms of biomass and EPS removal by any of the antimicrobial compounds.

The Friedman ranking test showed that overall, including all experimental assays, carvacrol was the least efficient antimicrobial/ anti-biofilm agent followed by trans-

cinnamaldehyde and thymol as the significantly ($P < 0.05$) most efficient antimicrobial towards both planktonic and biofilms cells.

4.4 The Effect of the Natural Compounds on the Inhibition (MBIC) and Eradication (MBEC) of Mixed Species Biofilm

Presence of all four natural antimicrobial compounds significantly ($P < 0.05$) inhibited biofilm formation of mixed species biofilm, composed of *L. monocytogenes 568* and a partner bacterium, by reducing the metabolic activity in biofilms as compared to the positive control (Table 2-A). The natural compounds did not equally effect the mixed biofilms, in terms of inhibition, as the required MBICs were dependent on the natural compounds and the bacterial species in the mixed biofilm (Table 2-A). The combination of *L. monocytogenes 568* and *S.* Typhimurium was the most susceptible mixture to trans-cinnamaldehyde with an MBIC of 0.09 mM and the most resistant to lemongrass EO with an MBIC of 0.06% (v/v) compared to the other two mixed biofilms (Table 2-A). The mixed biofilm of *L. monocytogenes 568* and *P. fluorescens* was the most resistant to thymol, carvacrol and trans-cinnamaldehyde compared to the two other mixtures with MBICs of 2 mM, 3 mM and 3 mM respectively (Table 2-A). *L. monocytogenes 568* and *S. baltica* was the most susceptible mixture to thymol with an MBIC of 0.50 mM compared to the two other mixtures (Table 2-A). Lemongrass EO was an effective mixed biofilm inhibitor at the two lowest concentrations tested (Table 2-A).

The natural compounds were not as effective in their ability to eradicate metabolic activity (MBEC) in pre-formed mixed species biofilm (Table 2-B). *L. monocytogenes 568* and *S.* Typhimurium was the only mixed biofilm where metabolic activity could be

Table 2. Minimum biofilm inhibitory concentration (MBIC) (A) and minimum biofilm eradication concentrations (MBEC) (B) of thymol, carvacrol, trans-cinnamaldehyde and lemongrass EO of the metabolic activity of *Listeria monocytogenes 568 (Lm 568)* mixed biofilms with *Salmonella enterica* Typhimurium (*Sme*), *Pseudomonas flurorescens (Pf)* or *Shewanella baltica (Shb)*. Values with > indicate that the compound was unable to eradicate the pre-formed mixed biofilm by 95% ± 5% at the highest tested concentration

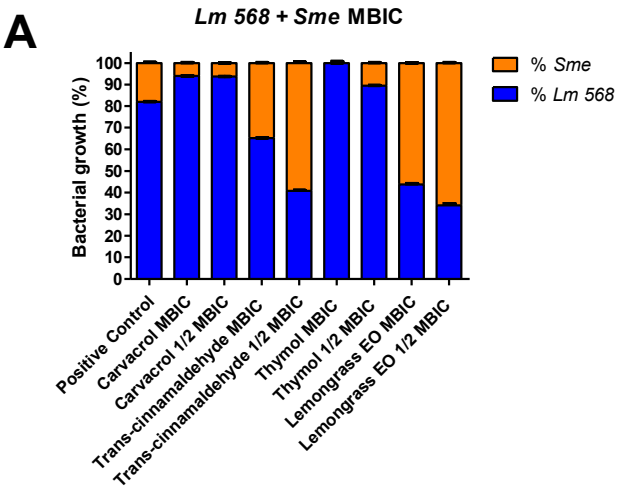
Treatments	Thymol (mM)	Carvacrol (mM)	Cinnamaldehyde (mM)	Lemongrass EO (% v/v)
A) MBIC of mixed species biofilm				
Lm 568 + Sme	1.00	1.50	0.09	0.06
Lm 568 + Pf	2.00	3.00	3.00	0.03
Lm 568 + Shb	0.50	1.50	0.75	0.03
B) MBEC of mixed species biofilm				
Lm 568 + Sme	2.00	6.00	12.00	2.00
Lm 568 + Pf	32.00	>24.00	>24.00	>16.00
Lm 568 + Shb	>32.00	6.00	>24.00	>16.00

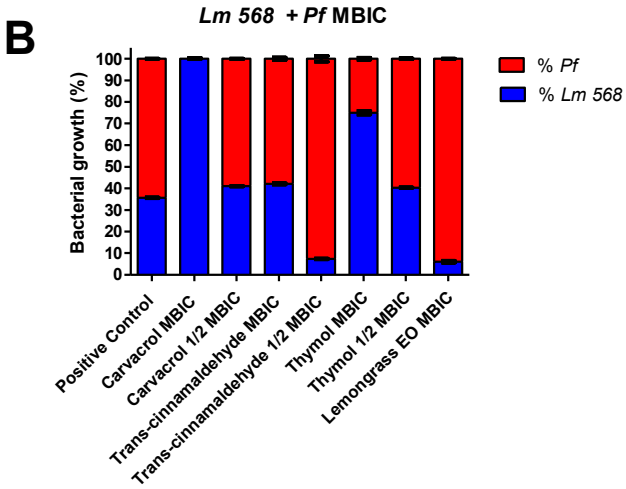
effectively reduced by 95% ± 5% by all four natural compounds, with MBECs ranging from 2 to 12 mM (Table 2-B). For *L. monocytogenes 568* and *P. fluorescens* mixed biofilm only thymol was able to eliminate activity in the mixed biofilm and it required the highest tested concentration of 32 mM (Table 2-B). The developed mixed biofilm of *L. monocytogenes 568* and *S. baltica* was only susceptible to carvacrol with an MBEC of 6 mM while showing resistance to the three other compounds (Table 2-B). In all cases, the MBIC for mixed species biofilms was lower than the MBEC of mixed biofilms, displaying one of the key findings that it required a higher or equal antimicrobial dosage to eradicate metabolic activity in preformed biofilm than to inhibit it in mixed species biofilms that were formed in the presence of the antimicrobials (Table 2).

4.5 Determination of Bacterial Species Proportions in Mixed Species Biofilm Inhibition Assays (MBIC)

The bacterial proportions in each mixed species biofilm were determined in the untreated biofilm (positive control), as well as in each of the mixed biofilms treated with the antimicrobial compounds at the metabolic MBICs and ½ MBICs levels derived from the MTT staining assays (Section 4.4). The proportions of each bacterium in the mixed biofilms were shown to be dependent on the bacterial mix, the natural antimicrobial treatment and its applied dosage.

In the mixed biofilm of *L. monocytogenes 568* and *S.* Typhimurium, it was observed that *L. monocytogenes 568* outcompeted *S.* Typhimurium to constitute 81.9% of the population in untreated biofilms (Figure 5-A). When treated with the MBIC and ½


A
Lm 568 + Sme MBIC
Bacterial growth (%)
% Sme
% Lm 568
Positive Control
Carvacrol MBIC
Carvacrol 1/2 MBIC
Trans-cinnamaldehyde MBIC
Trans-cinnamaldehyde 1/2 MBIC
Thymol MBIC
Thymol 1/2 MBIC
Lemongrass EO MBIC
Lemongrass EO 1/2 MBIC


B
Lm 568 + Pf MBIC
Bacterial growth (%)
% Pf
% Lm 568
Positive Control
Carvacrol MBIC
Carvacrol 1/2 MBIC
Trans-cinnamaldehyde MBIC
Trans-cinnamaldehyde 1/2 MBIC
Thymol MBIC
Thymol 1/2 MBIC
Lemongrass EO MBIC

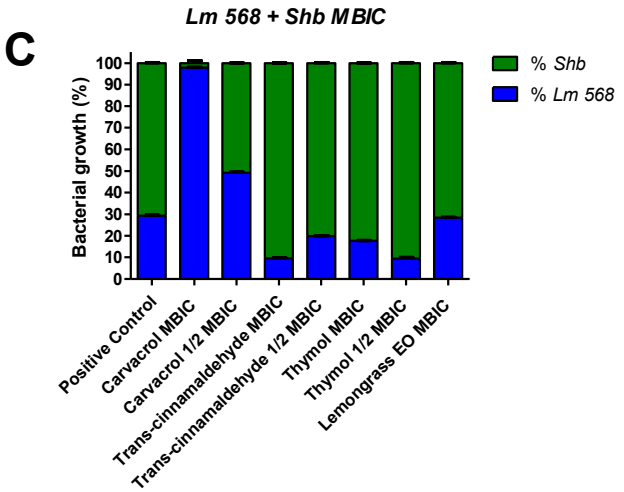

C
Lm 568 + Shb MBIC
Bacterial growth (%)
% Shb
% Lm 568
Positive Control
Carvacrol MBIC
Carvacrol 1/2 MBIC
Trans-cinnamaldehyde MBIC
Trans-cinnamaldehyde 1/2 MBIC
Thymol MBIC
Thymol 1/2 MBIC
Lemongrass EO MBIC

Figure 5. The relative proportion of each bacterial component expressed at the % (± SD) of the total population in mixed biofilms made by A) *Listeria monocytogenes 568* and *Salmonella enterica* Typhimurium *(Lm 568 + Sme)*, B) *Listeria monocytogenes 568* and *Pseudomonas fluorescens (Lm 568 + Pf)* and C) *Listeria monocytogenes 568* and *Shewanella baltica (Lm 568 + Shb)*. The proportions were determined for untreated (positive control) and treated mixed biofilms where biofilm formed in the presence of the mixed biofilm MBIC and ½ MBIC of carvacrol, trans-cinnamaldehyde, thymol and lemongrass EO using the MBIC values which were previously determined in MTT staining assays. The biofilm populations were enumerated on selective agars following incubation for 3 days at 15 °C.

MBIC of carvacrol the biofilm remained dominated by *L. monocytogenes 568,* now present at an even higher percentage of 93% (Figure 5-A). The trans-cinnamaldehyde treated mixed biofilm consisted of 65% *L. monocytogenes 568* at the MBIC concentration, which dropped to 40% *L. monocytogenes 568* when treated with one dilution lower (1/2 MBIC) of the compound (Figure 5-A). When the mixture of *L. monocytogenes 568* and *S.* Typhimurium was treated with thymol, the biofilm population consisted of 100% *L. monocytogenes 568* at the MBIC and 89.5% *L. monocytogenes 568* when treated with ½ MBIC (Figure 5-A). In contrast, application of the lemongrass EO treatment during the formation of the *L. monocytogenes 568* and *S.* Typhimurium mixed biofilm resulted in *S.* Typhimurium slightly outcompeting *L. monocytogenes 568* to make up 56% of the population (Figure 5-A). These results show that the proportions of the partner bacteria in the *Listeria* and *Salmonella* mixed biofilms vary depending on the treatment compound and the applied concentration.

The proportions in the untreated mixed biofilm of *L. monocytogenes 568* and *P. fluorescens* consisted of 64.4% *P. fluorescens* and 35.6% *L. monocytogenes 568* (Figure

5-B). When the mixed biofilm formed in the presence of the MBIC for carvacrol, it was composed of 100% *L. monocytogenes 568,* showing an increased susceptibility to carvacrol for *P. fluorescens* allowing *L. monocytogenes 568* to completely take over the biofilm (Figure 5-B). When treated with the ½ MBIC of carvacrol *P. fluorescens* was able to regain dominance to make up 59% of the mixed biofilm (Figure 5-B). The proportions of *L. monocytogenes 568* and *P. fluorescens* in biofilms exposed to trans-cinnamaldehyde during formation were relatively even at the MBIC with a slightly higher percentage of *P. fluorescens*, however, when treated with the ½ MBIC, *P. fluorescens* outcompeted *L. monocytogenes 568* to constitute 92% of the bacterial community (Figure 5-B). When treated with the thymol MBIC, the majority of the bacteria in the mixed *L. monocytogenes 568* and *P. fluorescens* biofilm consisted of *L. monocytogenes 568* (75%) (Figure 5-B). Once again it was observed that when treated below the MBIC level, the proportions in the biofilm changed and at the thymol ½ MBIC, *P. fluorescens* was able to grow to levels of 59% in the mixed biofilm (Figure 5-B). The lemongrass EO treated mixed biofilm consisted of 93% *P. fluorescens* (Figure 5-B). The MBIC for lemongrass was already at the lowest tested concentration preventing a ½ MBIC of being evaluated.

The composition of the mixed biofilm made by *L. monocytogenes 568* and *S. baltica* exhibited similar trends to those observed for the mixed biofilm of *L. monocytogenes 568* and *P. fluorescens* (Figure 5 B-C). The untreated, positive control, biofilm consisted of 70% *S. baltica* showing that *L. monocytogenes 568* was outcompeted when mixed with this spoilage organism (Figure 5-C). In contrast, when forming biofilm in the presence of carvacrol at the MBIC, *S. baltica* was outcompeted by *L. monocytogenes 568* that now made up 97% of the population (Figure 5-C). At the ½

MBIC of carvacrol, the proportions were close to equal for *L. monocytogenes 568* and *S. baltica* (Figure 5-C). The mixed biofilm consisted of 90% and 80% *S. baltica* when forming in the presence of the MBIC and ½ MBIC of trans-cinnamaldehyde, respectively (Figure 5-C). The proportions in the mixed biofilms made by *L. monocytogenes 568* and *S. baltica* when treated with thymol were 82% and 90% *S. baltica* at the thymol MBIC and thymol ½ MBIC, respectively (Figure 5-C). The lemongrass EO treated mixed biofilm consisted of 71% *S. baltica* when the MBIC for the natural compound was applied during biofilm formation (Figure 5-C).

4.6 Determination of Bacterial Species Proportions in Mixed species Biofilm Eradication Assays (MBEC)

The effect of the MBECs of thymol, carvacrol, trans-cinnamaldehyde and lemongrass EO on the population distribution after treatment of pre-formed mixed biofilms was also determined dependant on the mixture of bacteria, the natural antimicrobial treatment and the biofilm developmental stage.

The proportions of *L. monocytogenes 568* and *S.* Typhimurium in the preformed biofilm (3-day pre-formed biofilm, positive control at day 0 of the antimicrobial treatment period, T=0) was dominated by *L. monocytogenes 568* at 88.7% (Table 3-A). After incubation for an additional 2 days (T=2), *L. monocytogenes 568* remained the dominant bacterium but the proportion dropped to 66% showing that within this time frame, *S.* Typhimurium was able to survive in the highly *L. monocytogenes 568* populated environment and begin to increase its population in the biofilm. Addition of MBEC of carvacrol and lemongrass EO to the pre-formed biofilm followed by incubation

Table 3. The relative proportion of each bacteria, expressed at the % (± SD) of the total population, in mixed biofilms made by A) *Listeria monocytogenes* and *Salmonella enterica* Typhimurium (*Lm 568 + Sme)*, B) *Listeria monocytogenes* and *Pseudomonas fluorescens (Lm 568 + Pf* and C) *Listeria monocytogenes* and *Shewanella baltica (Lm 568 + Shb)*. The proportions were determined for the pre-formed untreated (positive control) after 3 days (T=0) and 5 days (T=2) incubation at 15 °C. Proportions were also determined for the 3 day pre-formed mixed biofilms, which were treated with the MBEC of carvacrol, trans-cinnamaldehyde, thymol and lemongrass EO and re-incubated for 2 days at 15 °C before testing. The MBECs were previously determined in MTT staining assays.* is indicative of treatments that yielded a bacterial population under the detection limit (< 1.55 log CFU/cm^2).

Treatments	***Lm 568* (%)**	***Lm 568* SD**	***Sme* (%)**	***Sme* SD**
A) Mixed Species Biofilm *Lm 568* + *Sme*				
Positive Control T=0	88.7	0.24	11.3	0.11
Positive Control T=2	66.0	0.14	34.0	0.29
Carvacrol	*	*	*	*
Trans-cinnamaldehyde	98.1	0.14	1.9	0.00
Thymol	>99.9	0.02	<0.1	0.92
Lemongrass EO	*	*	*	*
	***Lm 568* (%)**	***Lm 568* SD**	***Pf* (%)**	***Pf* SD**
B) Mixed Species Biofilm *Lm 568* + *Pf*				
Positive Control T=0	6.4	0.14	93.6	0.07
Positive Control T=2	39.7	0.04	60.3	0.31
Carvacrol	*	*	*	*
Trans-cinnamaldehyde	99.8	0.21	0.2	0.00
Thymol	*	*	*	*
Lemongrass EO	<0.1	0.00	>99.9	0.05
	***Lm 568* (%)**	***Lm 568* SD**	***Shb* (%)**	***Shb* SD**
C) Mixed Species Biofilm *Lm 568* + *Shb*				
Positive Control T=0	7.6	0.14	92.4	0.07
Positive Control T=2	94.7	0.19	5.3	0.19
Carvacrol	*	*	*	*
Trans-cinnamaldehyde	*	*	*	*
Thymol	*	*	*	*
Lemongrass EO	*	*	*	*

for 2 days caused the bacterial populations to drop below the detection limits (< 1.55 log CFU/cm^2) thus preventing proportions of viable cells in the mixed biofilm to be determined (Table 3-A). When the mixed biofilm of *L. monocytogenes 568* and *S.* Typhimurium was treated with trans-cinnamaldehyde and thymol, *L. monocytogenes 568* outcompeted *S.* Typhimurium by 98.1 and >99.9% respectively. In the case of thymol, viable numbers of *S.* Typhimurium decreased below the detection limit leading to complete domination by *L. monocytogenes 568* in the treated biofilms (Table 3-A).

The proportions in the pre-formed *L. monocytogenes 568* and *P. fluorescens* biofilm was dominated by *P. fluorescens* at 93.6% in the untreated 3-day old biofilms (positive control, T=0), however proportions were altered after two additional incubation days (T=2) when the *P. fluorescens* population was reduced to 60.3% (Table 3-B). When MBECs of carvacrol and thymol were applied to pre-formed *L. monocytogenes 568* and *P. fluorescens* mixed biofilm followed by a 2 day incubation period, it resulted in bacterial populations being reduced to below detected limits (<1.55 log CFU/cm^2), thus preventing determination of viable cell proportions in biofilms (Table 3-B). When the trans-cinnamaldehyde MBEC was applied to the pre-formed mixed biofilm, *L. monocytogenes 568* dominated the biofilm by 99.8%. (Table 3-B). In contrast, the lemongrass EO MBEC treated mixed biofilm consisted of >99.9% *P. fluorescens* (Table 3-B).

The proportions determined for the pre-formed biofilm made by *L. monocytogenes 568* and *S. baltica* were limited to the untreated positive controls. The addition of all four natural compounds at their MBEC to 3-day old pre-formed biofilm, followed by a 2-day incubation period resulted in a drop in the bacterial population below

the detection limit (< 1.55 log CFU/cm^2). The proportions determined for both development stages of the untreated positive control (T=0, T=2) showed a major contrast. The pre-formed *L. monocytogenes 568* and *S. baltica* mixed biofilm at 3 days incubation (T=0) was composed of 92.4% *S. baltica*, however, after an additional 2 days incubation (T=2) proportions shifted and *L. monocytogenes 568* dominated the mixed biofilm by 94% (Table 3-C).

4.7 Scanning Electron Microscopy (SEM) of Single Species Biofilms.

SEM images of untreated single species biofilms made by *L. monocytogenes 568, S.* Typhimurium*, S. baltica* and *P. fluorescens* following 3 days of incubation are shown in Figure 6. The untreated *L. monocytogenes 568* biofilm showed the least amount of biofilm formation (Figure 6-A). Only a few clusters of small rods were observed with single rods surrounding them. The *S.* Typhimurium biofilm was also sparse and appeared to cluster around crevices in the polystyrene surface (Figure 6-B). *S.* Typhimurium single cells were seen to attach themselves onto the surface using flagella or fibril structures (Figure 6-B). *S. baltica* biofilms were much more abundant and appeared in cluster-like forms with numerous surrounding singular rods (Figure 6-C). *P. fluorescens* biofilm appeared in aggregates with singular surrounding cells that were connected by unidentified materials, possibly EPS (Figure 6-D). These micrographs show that the spoilage bacteria *S. baltica* and *P. fluorescens* have greater biofilm forming abilities after 3 days of incubation at 15 °C. SEM images obtained after application of MBIC level treatments with the antimicrobials during formation of the 3-day single species biofilms revealed little to no growth (data not shown).

Due to the poor development of listerial biofilm after 3 days, which was to be expected based on previously obtained staining results, *L. monocytogenes 568* biofilms were allowed to form for 6 days in the presence of the natural compounds at their MBICs and pre-formed for 6 days prior to addition of natural compounds at their MBECs for comparison. The micrographs for the MBIC and MBEC treated biofilm matched the quantitative metabolic and biomass/EPS formation results. The untreated 6-day old *L. monocytogenes 568* biofilm showed a greater number of rods with evidence of attachment by fibril-like structures (Figure 7-A). In agreement with the quantitative staining data, each treated biofilm showed a reduced number of biofilm cells in comparison to the control, illustrating the biomass inhibitory effect of the natural antimicrobial compounds (Figure 7 A-E).

The eradication, defined as the complete physical removal of *L. monocytogenes 568* pre-formed biofilm after 6 days of incubation at 15 °C, was not achieved by any of the four natural compounds at the highest experimentally tested concentrations, which was subjected to SEM to visualize the effect on the biofilm. For the untreated positive control, large masses of cells entangled in unidentified materials, most likely EPS, were observed as well as several elongated and chain-like rods (Figure 8-A). Although none of the natural compounds removed the biomass to MBEC levels of 95% ± 5%, it is obvious that the biomass was greatly reduced by the addition of each natural compound (Figure 8 A-E). A greater reduction appears to take place when treated with carvacrol and the least reduction when treated with lemongrass EO, in agreement with previous quantitative findings (Figure 8 B-E).

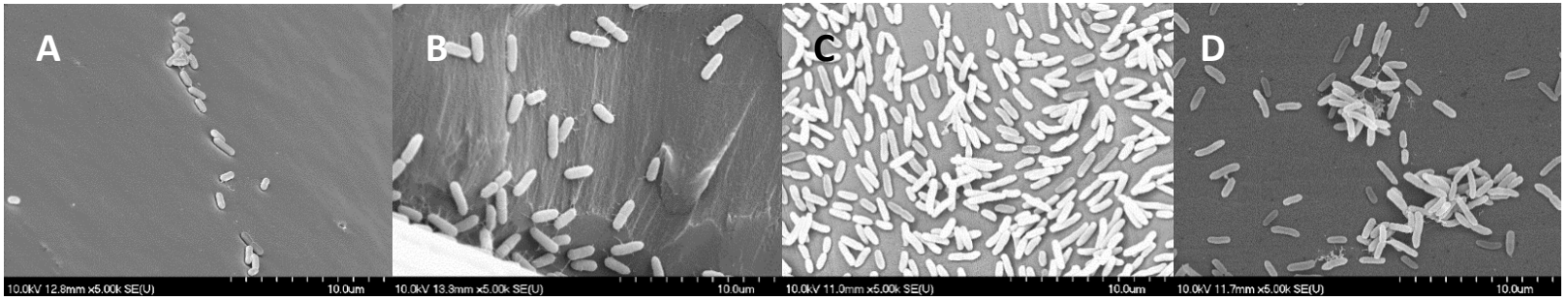


Figure 6. SEM micrographs of untreated positive control of biofilms formed for 3 days at 15 °C for A) *Listeria monocytogenes 568*, B) *Salmonella enterica* Typhimurium, C) *Shewanella baltica* and D) *Pseudomonas fluorescens* at 15 °C on polystyrene pegs. Scale 10 µm, V_{acce}: 10 kV.

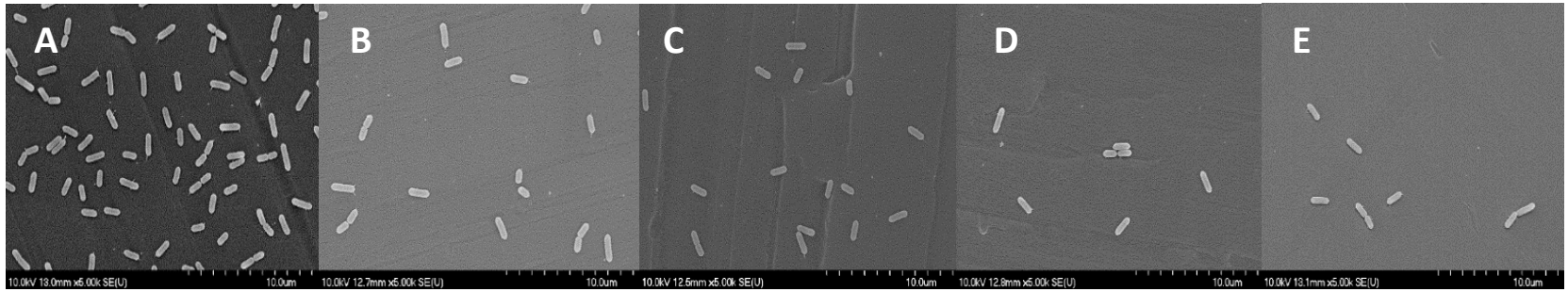


Figure 7. SEM micrographs of *Listeria monocytogenes 568* biofilm untreated control (A), and MBIC treated biofilm for B) carvacrol (0.045 mM), C) trans-cinnamaldehyde (0.045 mM), D) thymol (0.09 mM) and E) lemongrass EO (0.03% v/v) incubated for 6 days using MBICs obtained in CV staining assays. Scale 10 µm, V_{acce}: 10 kV.

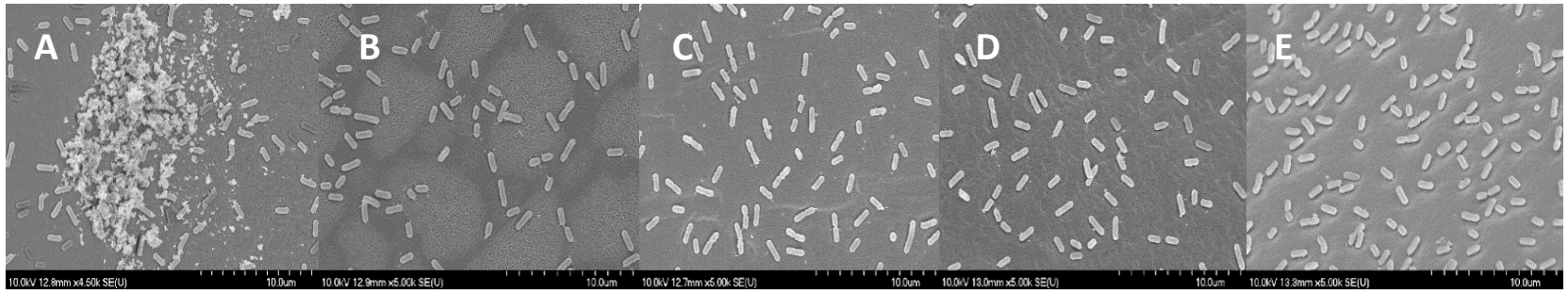


Figure 8. SEM micrographs of *Listeria monocytogenes 568* biofilm untreated control (A), and MBEC treated biofilm for B) carvacrol (24 mM), C) trans-cinnamaldehyde (24 mM), D) thymol (32 mM) and E) lemongrass EO (16% v/v) incubated for 6 days then treated for 2 days at 15 °C using MBECs obtained in CV staining assays. Scale 10 µm, V_{acce}: 10 kV.

4.8 Scanning Electron Microscopy (SEM) of Mixed Species Biofilms.

SEM images of mixed biofilms consisting of *L. monocytogenes 568* together with one of the partner bacteria, *S.* Typhimurium*, P. fluorescens* or *S. baltica*, were obtained for untreated and treated biofilms at the MBICs and MBECs of all four natural antimicrobial compounds. The micrograph for the untreated mixed 3-day old biofilm of *L. monocytogenes 568* and *S.* Typhimurium revealed a low amount of observable biofilm with several small clusters of rods scattered throughout the surface (Figure 9-A). When treated with the MBIC of carvacrol the mixed *L. monocytogenes 568* and *S.* Typhimurium biofilm contained a similar amount of cells than the control (Figure 9-B). For the trans-cinnamaldehyde, thymol and lemongrass EO treated mixed biofilm of *L. monocytogenes 568* and *S.* Typhimurium*,* there were small clusters of biomass but no rods were clearly observed in these masses (Figure 9 C-E). Although there were only a small amount of observable bacteria, the treated biofilms contained a reduced number of bacteria compared to the untreated (positive control) biofilm, confirming the previous results that these compounds significantly inhibit the formation of *L. monocytogenes 568* and *S.* Typhimurium mixed biofilm (Figure 9 A-E).

The mixed *L. monocytogenes 568* and *P. fluorescens* biofilm when left untreated formed large assemblages of biofilm, with bacterial rods connected by a mass of

biological material which may be EPS (Figure 10-A). When treated with the carvacrol MBIC, a reduction in both the number of rods and additional biological material surrounding the cells was observed (Figure 10-B). Similar observations were seen when the mixture was treated with trans-cinnamaldehyde (Figure 10-C). When treated with thymol, only small clusters of identifiable masses were observed with no rods clearly identifiable, showing the greater inhibition following treatment with thymol MBIC (Figure 10-D). For the lemongrass EO treated biofilms a reduced amount of scattered rods were observed without any additional connective materials (Figure 10-E). These micrographs further prove that the four natural compounds inhibit the formation of the mixed *L. monocytogenes 568* and *P. fluorescens* mixed biofilm.

The mixed biofilm of *L. monocytogenes 568* and *S. baltica* when left untreated formed biofilm that covered the surface area and was characterized by small bundles of rods (Figure 11-A). When the mixed biofilm was treated with the carvacrol MBIC a significant reduction in biofilm occurred, however, small clusters of rods with surrounding connective material were still observed (Figure 11-B). Similar results were observed when the mixed biofilm was treated with trans-cinnamaldehyde, although instead of clusters, a web-like structure was observed with entrapped bacterial rods and connective material (Figure 9-C). For mixed biofilm treated with the MBIC for thymol, no reduction of biomass was observed compared to the positive control (Figure 11-D). The surface area was covered with biofilm cells and in addition, large masses of connective material which were absent in the positive control (Figure 11-D). When the mixed biofilm was treated with the MBIC for lemongrass EO, a reduction in biofilm

population was observed although similarly to carvacrol, small clusters of connective biological material were present (Figure 11-E).

The effect of adding the antimicrobials at their MBEC on pre-formed mixed biofilms were also microscopically evaluated. The mixed biofilm micrographs for *L. monocytogenes 568* and *S.* Typhimurium followed results from quantitative assays showing a reduction of the pre-formed biofilm mass after treatment with all four natural compounds for 2 days as compared to the untreated positive control (Figure 12 A-E). The untreated control covered the surface area in a relatively equal spread of biofilm cells covered by an amorphous biological material (Figure 12-A). Treating the mixed biofilm with carvacrol reduced the number of rod-shape bacteria as well as the amorphous connective material (Figure 12-B). The trans-cinnamaldehyde treated mixed biofilm showed a reduction in biofilm, however, large groupings of connective EPS-like material remained along with some elongated chain-like rods (Figure 12-C). Similar results were observed when the mixed *L. monocytogenes 568* and *S.* Typhimurium biofilm was treated with the MBEC of thymol and lemongrass EO with a larger biofilm reduction observed for the latter antimicrobial (Figure 12 D-E).

The mixed biofilm made by *L. monocytogenes 568* and *P. fluorescens* showed resistance to the natural compounds in the eradication assay with the exception of thymol at its highest tested (MBEC) concentration. Although no significant removal was quantitatively obtained, the micrographs show a reduction of observable biomass (Figure 13 A-E). The untreated positive control showed large clusters of rods entrapped in a mass of EPS-like biological material connecting the cells (Figure 13-A). When treated with carvacrol the biofilm appeared to resemble the positive control in terms of population but

the rods appeared to be flattened and stretched compared to the plump and rounded rods in the positive control (Figure 13-B). When treated with trans-cinnamaldehyde a much greater reduction is observed with singular cells spread out across the surface with the occasional mass of amorphous biological material (Figure 13-C). The thymol treated mixed biofilm appeared to harbour slightly less singular cells but the same amount of connected biomass as the positive control (Figure 13-D). When treated with lemongrass EO both a reduced amount of biofilm and connective materials were observed (Figure 13-E).

The micrographs taken of *L. monocytogenes 568* and *S. baltica* mixed biofilms treated with the MBEC more closely matched the quantitative results. The untreated positive control for *L. monocytogenes* and *S. baltica* biofilm consisted of a heavily covered surface with layers of thick connective biological materials forming clusters of rods (Figure 14-A). As seen in the carvacrol treated mixed biofilm of *L. monocytogenes 568* and *P. fluorescens*, a reduction of biofilm mass was observed along with the appearance of flattened and stretched cells (Figure 14-B). A greater reduction was seen when treated with carvacrol compared to the other treatments. When treated with the MBEC for trans-cinnamaldehyde, the mixed biofilm showed a slight reduction in biofilm cells and was characterized by a more web-like structure connecting the rods (Figure 14-C). The singular cells were also observed to look depleted and deformed compared to the positive control. The micrographs for the mixed biofilm treated with thymol and lemongrass EO show similar web-like clusters of cells wrapped into connective biological material (Figure 14 D-E). The singular cells for these two treatments also look flattened and stretched compared to the positive control (Figure 14 D-E).

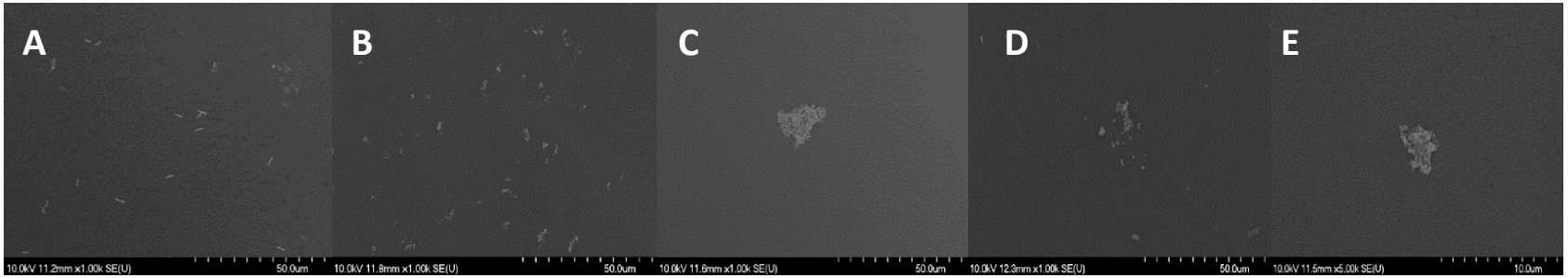


Figure 9. SEM micrographs of *Listeria monocytogenes 568* and *Salmonella enterica* Typhimurium mixed biofilm untreated control (A), and MBIC treated biofilm for B) carvacrol (1.5 mM), C) trans-cinnamaldehyde (0.09 mM), D) thymol (1 mM) and E) lemongrass EO (0.06% v/v) incubated for 3 days using MBICs obtained in MTT staining assays. Scale 10 µm (E) and 50 µm (A-D), V_{acce}: 10 kV.

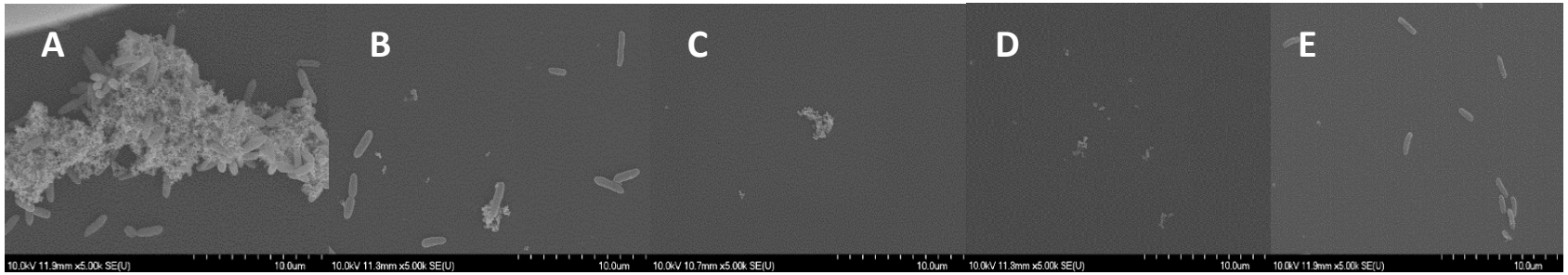


Figure 10. SEM micrographs of *Listeria monocytogenes 568* and *Pseudomonas fluorescens* mixed biofilm untreated control (A), and MBIC treated biofilm for B) carvacrol (3 mM), C) trans-cinnamaldehyde (3 mM), D) thymol (2 mM) and E) lemongrass EO (0.03% v/v) incubated for 3 days using MBICs obtained in MTT staining assays. Scale 10 µm, V_{acce}: 10 kV.

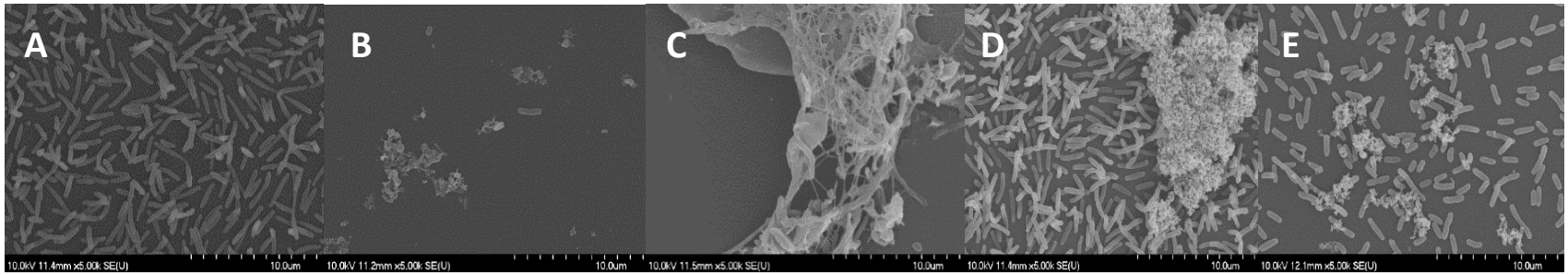


Figure 11. SEM micrographs of *Listeria monocytogenes 568* and *Shewanella baltica* mixed biofilm untreated control (A), and MBIC treated biofilm for B) carvacrol (1.5 mM), C) trans-cinnamaldehyde (0.75 mM), D) thymol (0.5 mM) and E) lemongrass EO (0.03% v/v) incubated for 3 days using MBICs obtained in MTT staining assays. Scale 10 µm, V_{acce}: 10 kV

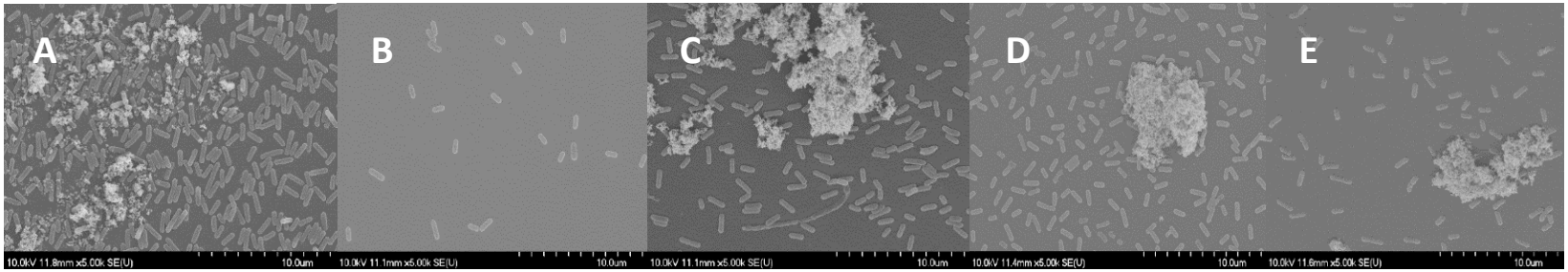


Figure 12. SEM micrographs of *Listeria monocytogenes 568* and *Salmonella enterica* Typhimurium mixed biofilm untreated control (A), and MBEC treated biofilm for B) carvacrol (6 mM), C) trans-cinnamaldehyde (12 mM), D) thymol (2 mM) and E) lemongrass EO (2% v/v) incubated for 3 days using MBECs obtained in MTT staining assays. Scale 10 µm, V_{acce}: 10 kV.

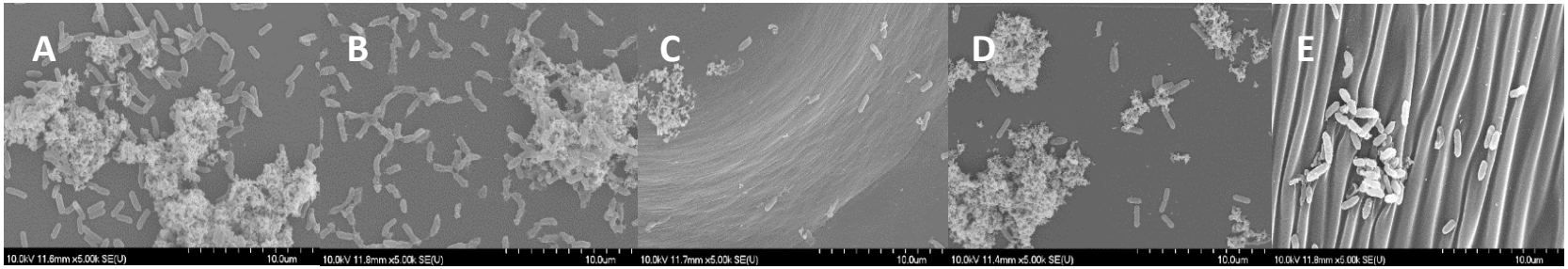


Figure 13. SEM micrographs of *Listeria monocytogenes 568* and *Pseudomonas fluorescens* mixed biofilm untreated control (A), and MBEC treated biofilm for B) carvacrol (24 mM), C) trans-cinnamaldehyde (24 mM), D) thymol (32 mM) and E) lemongrass EO (16% v/v) incubated for 3 days using MBECs obtained in MTT staining assays. Scale 10 µm, V_{acce}: 10 kV.

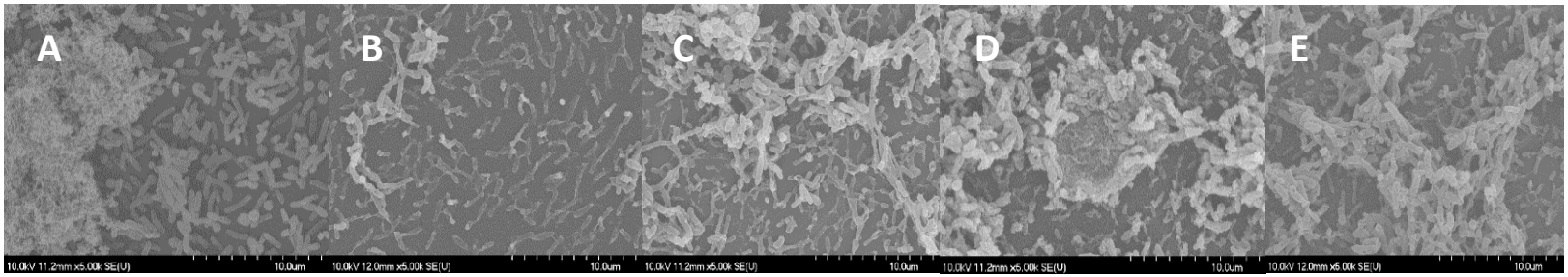


Figure 14. SEM micrographs of *Listeria monocytogenes 568* and *Shewanella baltica* mixed biofilm untreated control (A), and MBEC treated biofilm for B) carvacrol (6 mM), C) trans-cinnamaldehyde (24 mM), D) thymol (32 mM) and E) lemongrass EO (16% v/v) incubated for 3 days using MBECs obtained in MTT staining assays. Scale 10 μm, V_{acce}: 10 kV.

CHAPTER 5 DISCUSSION

5.1 The Effect of the Natural Antimicrobial Compounds on Bacterial Planktonic Growth (MIC and MBC)

All four natural compounds; thymol, carvacrol, trans-cinnamaldehyde and lemongrass EO were able to significantly ($P < 0.05$) inhibit and eradicate the planktonic growth of *L. monocytogenes, S.* Typhimurium*, P. fluorescens, S. baltica* and *St. aureus*. As expected, a higher concentration of the natural antimicrobial compounds was required to eradicate the bacteria than to inhibit its growth. Similar findings have been previously observed in a study by Upadhyay et al. (2013) that tested carvacrol, trans-cinnamaldehyde and thymol against *L. monocytogenes* planktonic growth at 37 °C for 24 hours. Jadhav et al. (2013) studied the effects of yarrow EO on *L. monocytogenes* planktonic cells at 37 °C for 24 hours and found the MBC for *L. monocytogenes* cells to require a higher dosage than the MIC. Adukwu et al. (2012) tested the effects of lemongrass EO on *St. aureus* planktonic cells at 37 °C for 24 hours, which resulted in similar findings with the MBC requiring higher concentrations. The MIC results found in the current study for *St. aureus* was similar to findings by Adukwu et al. (2012), whereas in contrast, the MBC from the previous study was much lower than the MBC of 8% (v/v) found for *St. aureus* treated with lemongrass EO in the current study. This could be due to use of different strains and differences in experimental conditions, including different assay temperatures of 15 vs. 37 °C.

Naik et al. (2010) studied the effect of lemongrass EO against several pathogenic bacteria at 37 °C for 24 to 48 hours and found that lemongrass EO was inhibitory to bacterial growth at very low concentrations. The study also showed similar results to

those found in the present study where the MBC was equal or higher than the MIC and ranged from 0.06 – 0.50% (v/v). The same trend was also found in a study by Nostro et al. (2007) when the effects of carvacrol and thymol on *St. aureus* planktonic growth were evaluated.

The natural antimicrobial compounds' effect on the planktonic bacteria depended on the antimicrobial compounds themselves, their dosage and the treated microorganism. *S. baltica* was significantly ($P < 0.05$) more susceptible to all treatments in planktonic inhibition assays compared to the other bacteria followed by *S.* Typhimurium. *S. baltica* has been found to have slower growth rates at 15 °C than *L. monocytogenes* and *P. fluorescens* (Daneshvar Alavi and Truelstrup Hansen 2013), which could explain its higher susceptibility. For thymol and carvacrol it appears that Gram-positive bacteria (*L. monocytogenes* and *St. aureus*) have slightly higher MICs than the Gram-negatives but there is not a great difference among the MBC values. Thymol and carvacrol are able to disintegrate the outer membrane of Gram-negative bacteria and release lipopolysaccharides (Burt 2004), which may have increased their ability to inhibit Gram-negative bacteria at lower concentrations. For trans-cinnamaldehyde and lemongrass EO there is no significant trend that could be related to cell wall properties, i.e., Gram-negative vs. Gram-positive.

5.2 The Effect of the Natural Antimicrobial Compounds on the Inhibition of Biofilm Formation (MBIC)

The biofilm inhibition assay successfully showed that thymol, carvacrol, trans-cinnamaldehyde and lemongrass EO were able to inhibit ($P < 0.05$) formation of biofilms

in terms of their metabolic activity (MTT stain), and their biofilm biomass measured as CV or safranin stainable EPS for all bacterial species. MTT is a tetrazolium based dye that measures the remaining active metabolism by bacteria in the biofilm following disinfection treatments (Molecular Probes Inc. 2002; Pitts et al. 2003). CV is a dye that measures the amount of biofilm or biomass but not its activity, hence it measures removal of cells and/or the biofilm amorphous material (EPS) but not disinfection (Pitts et al. 2003).

The results showed that the natural antimicrobial compounds' effect was dose dependent and MBICs varied among the tested bacterial species as well as staining assays. Generally, it was observed that a higher concentration of natural antimicrobial compounds was required to inhibit the metabolic activity, hence kill the bacteria, than to inhibit the formation of biomass such as EPS. This suggests that the presence of the antimicrobials at low concentrations may inhibit the biofilms' ability to form EPS, however, bacteria will remain viable, potentially allowing them to persist in food processing environments.

In some cases such as when *L. monocytogenes 568* was treated with carvacrol, it was the most resistant bacterium with respect to inhibition of metabolic activity with an MBIC of 6 mM but the most susceptible bacteria when it came to inhibition of its biomass production with an MBIC at the lowest tested concentration of 0.045 mM. This suggested weak EPS forming abilities, but an increased resistance to carvacrol when it came to repression of metabolic activity in *L. monocytogenes 568* biofilm cells. SEM micrographs of *L. monocytogenes 568* treated with the MBIC of carvacrol for inhibition of biomass showed a reduction in the number of biofilm cells compared to the positive

control, and no visible EPS. In fact, EPS was absent in all MBIC treated *L. monocytogenes 568* biofilm micrographs. When Vasquez-Sanchez et al. (2013) tested BAC against *St. aureus* biofilms they found that for some strains there was no correlation between BAC resistance and biomass production with the possible reasons being due to a variation in expression of genes involved in biofilm production. This variation of genes could lead to differences in structure and composition of EPS along with variation in stability and resistance to treatments.

Trans-cinnamaldehyde was the most effective compound compared to carvacrol and thymol, requiring lower concentrations to inhibit biofilm formation with the exception of *P. fluorescens.* For the inhibition of metabolic activity, *P. fluorescens* was the most resistant to trans-cinnamaldehyde compared to all other bacteria; this spoilage organism is known for its strong biofilm forming abilities. (Daneshvar Alavi and Truelstrup Hansen 2013; Giaouris et al. 2013). Lemongrass EO was also a very effective antimicrobial; *L. monocytogenes 568, L. monocytogenes CP45-1*, *S. baltica* and *St. aureus* were all inhibited in terms of metabolic activity at the lowest tested concentration of 0.03% (v/v). For the inhibition of biomass formation the lowest concentration tested was the MBIC for all bacteria except *P. fluorescens* which required one dilution factor higher. In many instances, the lowest tested concentration was the MBIC for both staining methods. SEM micrographs of *L. monocytogenes 568* biofilm treated with the MBIC for lemongrass EO obtained for biomass inhibition showed greater reduction in biofilm cells compared to the positive control than the other compounds. Adukwu et al. (2012) also found lemongrass EO to be an effective antimicrobial at low concentrations, where when tested against *St. aureus* biofilms for 24 hours at 37 °C, MBICs ranged from

0.06 - 0.125% (v/v) for inhibition of biomass. A possible reason for lemongrass EO's greater ability to inhibit biofilm growth may be due to its different components. EO components are known to act synergistically so the EO will elicit a greater antimicrobial effect then its individual components on their own.

When treated with carvacrol, the Gram-positive bacteria required lower concentrations of the antimicrobial to inhibit the formation of biomass than the Gram-negative bacteria with the exception of *S. baltica.* Burt (2004) had previously stated that generally for EOs, Gram-negative microorganisms are slightly more resistant than Gram-positives which could be expected since Gram-negatives possess an outer membrane surrounding the cell wall which is absent in Gram-positives.

When comparing the MTT staining assay MBICs to the MICs, results showed that the metabolic MBICs were higher than or equal to the MIC for all antimicrobial compound and bacterial combinations. These results revealed that the biofilm cells were more resistant to reductions in their respiration than planktonic cells when grown in the presence of thymol, carvacrol, trans-cinnamaldehyde and lemongrass EO. Adukwu et al. (2012) evaluated the antimicrobial properties of lemongrass EO on *St. aureus* planktonic cells and biofilm and found that to prevent biofilm formation, twice the required concentration to inhibit planktonic cells was required. In agreement to these results, Vasquez-Sanchez et al. (2013) also observed an increased resistance of *St. aureus* biofilms compared to its planktonic counterparts when treated with commonly used chemical disinfectants, BAC, PAA and NaClO. Van der Veen and Abee (2011) tested BAC and PAA against *L. monocytogenes* planktonic and biofilm cells and also found that biofilms showed higher resistance to the treatments than planktonic cells. The

antimicrobial effect of carvacrol and thymol on *St. aureus* planktonic cells and biofilm was tested and also observed to require concentrations twofold or fourfold greater for inhibition of biofilm than for planktonic cell growth (Nostro et al. 2007). Kwiencinski et al. (2009) similarly found that tea tree oil at a concentration of 1% (v/v) was able to inhibit *St. aureus* biofilms. This concentration was two times greater than the concentration required to inhibit planktonic cells.

The increased resistance of biofilms to the natural antimicrobial compounds compared to planktonic cells has been attributed to their ability to form EPS. The EPS forms multiple layers surrounding and protecting the bacteria in a biofilm and allows it to attach to surfaces (Richards and Melander 2009; Simoes et al. 2010; Bridier et al. 2011). The EPS is not easily penetrable, irreversibly attached and may diffuse, degrade or trap the antimicrobial before it reaches the target organism (Richards and Melander 2009; Sandasi et al. 2010; Simoes et al. 2010; Bridier et al. 2011). If there is a heavy presence of EPS continuously protecting the bacteria that is being treated with diffused and now sub-inhibitory concentrations, it could lead to an increased bacterial resistance to those antimicrobials.

All the tested natural antimicrobial compounds prevented planktonic cells of forming irreversible resistant biofilm, proving to be a useful approach to control microbial adhesion and biofilm formation in food processing environments. Preventing biofilm formation and cell attachment is a more efficient control mechanism when dealing with biofilms in food processing environments than having to remove and disrupt an already formed biofilm. Biofilm formation is a survival mechanism but also contributes to its virulence, and persistence in food processing environments (Simoes et

al. 2010). The required concentration to inhibit formation of biofilm was dependent on the bacteria and natural antimicrobial compound. In agreement, Pitts et al. (2003) tested the efficacy of commonly used disinfectants against biofilms formed by *P. aeruginosa* and *St. epidermidis* and stated that biofilm reductions are microorganism and antimicrobial agent specific.

5.3 The Effect of the Natural Antimicrobial Compounds on the Eradication and Removal of Pre-formed Biofilms (MBEC)

The four natural antimicrobial compounds; thymol, carvacrol, trans-cinnamaldehyde and lemongrass EO were tested for their anti-biofilm properties against pre-formed and established biofilms of three maturities; low (10^3 CFU/mL initial concentration), intermediate (10^5 CFU/mL initial concentration) and high (10^7 CFU/mL initial concentration). The MBECs obtained depended on the natural antimicrobial compounds, the bacterial species and the development stage of the biofilm. Upadhyay et al. (2013) have previously found that carvacrol, thymol and trans-cinnamaldehyde constituted effective anti-biofilm treatments and inactivated pre-formed *L. monocytogenes* biofilms. Concentrations required to reduce the surviving population of *L. monocytogenes* biofilms were 5.0 and 10.0 mM for carvacrol and trans-cinnamaldehyde, and 3.3 and 5.0 mM for thymol on polystyrene and SS surfaces, respectively. With the exception of trans-cinnamaldehyde similar MBEC values were found for thymol and carvacrol in the current study. Differences could be due to the use of different strains, incubation period and temperature of incubation.

An increased tolerance to the natural antimicrobial compounds was generally observed as the biofilm increased in maturity. There were a few exceptions, where in

some cases a lower concentration of the antimicrobial was able to reduce high maturity biofilms (10^7 CFU/mL) at a level of ≥ 95% ± 5% than intermediary (10^5 CFU/mL) biofilms. This could be due to the aging bacteria in high maturity biofilms dying on their own, thus creating a more susceptible biofilm. These results show that the maturity of the biofilm affects the natural antimicrobial compounds' ability to eradicate the biofilm and disinfect the environment. These results also show the importance of using appropriate antimicrobial concentrations to prevent biofilms of maturing, spreading and possibly resisting sanitation and disinfection regimes. This assay simulated a worst case scenario that could occur in food processing environments such as leaving inadequately cleaned and disinfected surfaces over a longer time period, such as a weekend, allowing biofilm to mature.

Vasquez-Sanchez et al. (2013) studied the efficacy of BAC, a commonly used disinfectant, against *St. aureus* biofilms that had been incubated for 5, 24, 48 and 168 hours at 25 °C and found that *St. aureus* biofilm resistance to BAC increased as the biofilm matured. Kwiencinski et al. (2009) found that *St. aureus* in the stationary phase was more tolerant to tea tree oil than the bacteria in the exponential phase showing that as the bacteria move through the development stages it becomes more resistant. The study explained that as cells enter the stationary phase the cell membrane changes in terms of composition, fluidity, hydrophobicity and charge and since the cell membrane is the main target for EO compounds, these changes can account for the increase in tolerance. When the cells are both lodged in biofilm and in the stationary phase their tolerance increased greatly, which may be due to increased EPS production and alterations to the cell wall

allowing for the cells to become what is known as "persister cells" and resist control strategies.

Saa Ibusquiza et al. (2010) studied *L. monocytogenes* strains and the effect of BAC on *L. monocytogenes* biofilms. Their results, similar to those reported in the present study, varied among strains. The biofilms were formed for 4 and 11 days at 25 °C prior to treatment. One of the *L. monocytogenes* strains showed no difference in resistance while two other strains increased in resistance as the biofilm matured. The strains were also treated with PAA and nisin and showed differences in resistance, highlighting that the effect is dependent on the treatment compound as well as the bacteria. A study investigating the desiccation survival of *L. monocytogenes 568* in biofilms of three different maturities found that resistance to desiccation increased as the biofilm matured as well (Hingston et al. 2013).

Thymol and carvacrol were the most effective compounds, as these compounds were able to eradicate the metabolic activity of biofilm for a wider range of bacteria and biofilm maturities than the other compounds. Both were able to eradicate the same number of samples with thymol requiring slightly lower concentrations. This result would be expected, as thymol and carvacrol are structurally similar with the only difference being the position of the hydroxyl group and also share the same inhibitory mode of action (Burt 2004, Hyldgaard et al. 2012). However, the two most effective compounds in regard to inhibition of biofilm formation (MBIC), trans-cinnamaldehyde and lemongrass EO, were the least effective in terms of being able to eradicate metabolic activity in pre-formed biofilms. Only *St. aureus* and *P. fluorescens* were eradicated in biofilms of all three maturities following treatment with trans-cinnamaldehyde and

lemongrass EO. For trans-cinnamaldehyde, even application of the highest concentration was not able to eradicate metabolic activity in *S. baltica* biofilms regardless of the maturity and the Gram-positives were only eradicated when found in biofilm of the lowest maturity. For lemongrass EO, even the highest tested concentration was unable to eradicate metabolic activity of *L. monocytogenes 568* in biofilms of all development stages.

These results reveal that Gram-positives are more resistant for the eradication of biofilm by trans-cinnamaldehyde and lemongrass EO than Gram-negatives. While in comparison for thymol and carvacrol the only bacteria that were not eradicated in biofilms of all maturities were Gram-negatives. This suggests that the susceptibility of Gram-positives versus Gram-negatives is dependent on the natural antimicrobial compound which was also previously stated by Burt (2004). It appears that trans-cinnamaldehyde and lemongrass EO are more effective as antimicrobials and inhibiting biofilm formation than removing pre-formed biofilm while thymol and carvacrol are effective antimicrobials as well as anti-biofilm agents. Statistically, carvacrol was the least efficient antimicrobial/ anti-biofilm agent followed by trans-cinnamaldehyde and thymol. This rank based test included all inhibition and eradication results. Although trans-cinnamaldehyde was more efficient as an inhibitor of planktonic growth and biofilm, carvacrol was able to remove established biofilm making it a more ideal compound to use in food processing environments. Adukwu et al. (2012) found that lemongrass EO prevented *St. aureus* biofilm formation at low concentrations but was unable to remove already formed biofilms which agrees with the findings of this study. For some bacteria, pre-forming the biofilm resulted in drastic increases in their ability to

resist the antimicrobials compared to the MBIC assay. *S. baltica* was the most susceptible with respect to inhibition of biofilm formation (MBIC) but showed the most resistance to eradication of pre-formed biofilm while opposite results are seen in *S.* Typhimurium and *P. fluorescens*. This highlights that effective concentrations of natural antimicrobial compounds are affected by the biofilm development stage and the bacteria themselves.

It has also been observed that cells in established biofilm are more resistant to the natural antimicrobial compounds than cells involved in biofilm formation in the presence of antimicrobial compounds. In all cases, the MBECs were higher or equal to the MBIC values for the inhibition and eradication of metabolic activity of bacterial cells. Larger differences between these two results were seen in trans-cinnamaldehyde and lemongrass EO treatments due to their lack of ability to eradicate biofilm and strong ability to inhibit the formation of biofilm. A pre-formed matured biofilm would have had the opportunity to form strong irreversibly attached EPS surrounding cells thus preventing the antimicrobials from reaching the target microorganism. This was observed in the SEM micrographs where pre-formed biofilm was characterized by large aggregates of cells trapped in masses of biological material which were mostly absent in the biofilm formed in the presence of antimicrobial compounds. The cells also grew to stationary phase without any disruptions from treatments allowing them to proliferate and become more challenging for the natural antimicrobial compounds.

This trend was also reported by Jadhav et al. (2013), who tested the effect of yarrow EO for inhibition of biofilm formation as well as on pre-formed biofilm and found that a higher concentration of yarrow EO was required to eradicate pre-formed biofilms than to inhibit biofilm formation. The study explained possible reasons for this

outcome being the production of EPS and that most antimicrobial compounds are more effective against actively growing cells and that cells in a biofilm have poor growth rates due to lack of nutrients and oxygen. Nostro et al. (2007) evaluated the effect of thymol, carvacrol and oregano oil on *St. aureus* biofilms and found that a higher or equal concentration of all three treatments were required to eradicate the pre-formed biofilm than to inhibit biofilm formation, but all compounds were effective as anti-biofilm treatments.

The natural antimicrobial compounds have shown to effectively kill the sessile biofilm bacteria depending on their species, natural compound and maturity, but will not necessarily detach the cells or remove the biomass such as the EPS surrounding the biofilm. This could be due to a large mass of dead cells which were no longer viable but still remained trapped in the amorphous biofilm matrix. Attempts to determine the MBEC required for removal of biomass using CV and safranin staining had poor outcomes where removal and/or dispersal of biomass was only observed for Gram-negative bacteria treated with certain natural antimicrobials (data not shown). For example, carvacrol and trans-cinnamaldehyde were able to remove *P. fluorescens* biomass from pre-formed biofilm at all three development stages while carvacrol and thymol were able to do the same for *S.* Typhimurium. All four antimicrobial compounds were able to remove the biomass of *S. baltica* at low and intermediate maturities (10^3 and 10^5 CFU/mL) but not at the highest maturity (10^7 CFU/mL). *P. fluorescens* and *S. baltica* were strong and fast biofilm formers and had successful staining results after 2 days incubation at 15 °C while all other bacteria took 6 days incubation at 15 °C to yield successful staining results. This could explain why the treatments were able to remove the biomass; the bacteria grew

much faster than the rest but had less time to produce a thick layer of biomass that would protect them from the treatments. If biofilms of the two fast growing spoilage bacteria had been incubated for 6 days like the others, the staining assays may not have been successful due to an excessive amount of growth causing unreadable amounts of stained bacteria.

S. Typhimurium was incubated for 6 days prior to staining and was still able to be reduced in terms of its biofilm biomass. *S.* Typhimurium was highly susceptible scoring low MBECs for reduction of metabolic activity and was also the only species to obtain MBECs for all treatments at all biofilm developmental stages. These results suggests that *S.* Typhimurium's biofilm forming abilities are limited and the bacteria is easily eradicated and removed. In agreement, SEM micrographs of a *S.* Typhimurium 3 day biofilm and when mixed with *L. monocytogenes 568* showed a lesser amount of observable biofilm than other bacteria and mixed biofilm combinations. Pitts et al. (2003) tested anti-biofilm properties of commonly used disinfectants against *P. aeruginosa* and *St. epidermidis* using CTC metabolic and CV biomass staining and found that the percentage reduction obtained with CTC staining was larger than the percentage reduction acquired with CV staining. The study explained that the result was as expected since cells that are dead and/or removed cannot account for metabolic activity while dead cells can still account for biomass.

For the MIC, MBC, MBIC and MBEC determinations three strains of *L. monocytogenes* were used: *L. monocytogenes 085578* a clinical strain, *L. monocytogenes 568* a food processing related strain and *L. monocytogenes CP45-1* an environmental strain isolated from the Lake Fletcher watershed in Nova Scotia. Interestingly it was

observed for both planktonic and biofilm inhibition and eradication assays that the strains varied in their resistance to treatment with all four natural antimicrobial compounds. These results show that required dosages to inhibit and eliminate the bacteria in planktonic and biofilm form depends not only on the bacterial species but also the strain. Therefore, it is important when testing efficacy of disinfection treatments to use a library of strains of each bacterium instead of just one strain since some strains show higher resistance. In this case further work could be done on other strains of *S.* Typhimurium*, P. fluorescens, S. baltica* and *St. aureus* to ensure proper minimal concentrations have been identified. Vasquez-Sanchez et al. (2013) similarly observed variations in biofilm resistance among 26 *St. aureus* strains, where the resistance of each bacterium followed a different order for each tested biocide. The author explained the importance of testing several strains belonging to the same species when assessing the antimicrobial properties of a compound to ensure they are correctly applied and are able to effectively control the growth of all strains. Heir et al. (2004) tested 112 strains of *L. monocytogenes* against BAC and results varied greatly with sensitive strains and resistant strains requiring lower and higher concentrations. Rieu et al. (2008) evaluated six environmental and one culture collection *St. aureus* isolates for their abilities to form single and mixed species biofilms with *L. monocytogenes EGD-e* on SS coupons. The isolates differed in their ability to form single species biofilms. The effect of *L. monocytogenes EGD-e's* presence was also strain dependent with some strains decreasing in the mixed biofilm population while others increased.

5.4 The Effect of the Natural Antimicrobial Compounds on the Inhibition and Eradication of Mixed Species Biofilm

The four natural antimicrobial compounds were tested against mixed dual-species biofilms consisting of *L. monocytogenes* together with *S.* Typhimurium, *S. baltica* or *P. fluorescens*. All four compounds were able to significantly ($P < 0.05$) inhibit the formation of mixed biofilm, however, the eradication of the mixed species biofilm required higher concentrations or the treatments were unable to eradicate the biofilm at the highest tested concentration. These results follow the same trends as those obtained for single species assays where a higher concentration of antimicrobial was required to remove pre-formed biofilm than to inhibit its formation. As stated with single species biofilm, these differences could be due to an increased amount of EPS in the pre-formed and established biofilm, allowing the microorganism to be protected. In agreement, visualization by SEM showed that the established mixed biofilms consisted of a layer of biofilm cells with large clusters of biological material in some treated and untreated biofilms. In comparison, the mixed biofilm formed in the presence of treatment showed a reduction in visible biofilm and EPS-like structures. The EPS formation may also be enhanced in mixed species biofilms due to chemical interactions between polymers of each species leading to a more viscous biomass that prevents penetration of disinfection compounds (Bridier et al. 2011).

Most previous studies of mixed biofilms did not compare the inhibition of biofilm formation in the presence of antimicrobials to the removal of pre-formed mixed biofilm. Knowles et al. (2005) tested the efficacy of carvacrol applied as continuous pulses of low concentrations to mixed species biofilms of *St. aureus* and *S. enterica*. Carvacrol was able to inhibit biofilm formation of each bacterium in the early biofilm development

stages and prevented the dual species biofilm from maturing. Further exploration into the inhibition of mixed biofilm should be done. This could involve studying the EPS formation, composition and prevention strategies since it has proven to be the main factor in resistance of pre-formed and mature biofilms.

The MBICs and MBECs were dependent on the bacterial mixture as well as the natural antimicrobial compound and varied for each combination of the two. For the MBIC determination, low concentrations were required to inhibit the formation of biofilm for all mixtures. The natural antimicrobial compounds were not as effective for the eradication of pre-formed biofilm, only the mixed biofilm of *L. monocytogenes 568* and *S.* Typhimurium was eradicated by all four compounds. A visible reduction of the mixed species biofilms was observed by SEM following treatment with all four antimicrobial compounds as compared to the positive untreated control. Both *L. monocytogenes 568* and *S.* Typhimurium were eradicated as single species biofilms by all four antimicrobial treatments suggesting that mixing *L. monocytogenes 568* with *S.* Typhimurium did not greatly increase biofilm resistance. The mixture of *L. monocytogenes 568* and *P. fluorescens* was only eradicated by thymol at the highest tested concentration; as a single biofilm *P. fluorescens* was eradicated by all antimicrobial compounds suggesting an increased resistance when forming a mixed biofilm with *L. monocytogenes 568*. A visible reduction in cells but not additional connective material was observed by SEM for *L. monocytogenes 568* and *P. fluorescens* mixed biofilms after treatment with thymol showing that the compound does not disperse the biofilm or its supplementary materials. The mixed biofilm of *L. monocytogenes 568* and *S. baltica* was eradicated by carvacrol only, while in single species biofilm *S. baltica*

was eliminated by thymol, carvacrol and lemongrass EO indicating an increase in resistance to the natural antimicrobials. The SEM micrographs of *L. monocytogenes 568* and *S. baltica* followed the quantitative findings, and a reduction in biofilm and biomass was observed in the carvacrol treated mixed biofilm only. In terms of inhibition and eradication, mixed species biofilm were not equally affected by thymol, carvacrol, trans-cinnamaldehyde and lemongrass EO. To completely remove a mixed biofilm, higher natural antimicrobial compound concentrations were required.

The four antimicrobials were not equally effective when comparing their effect on single and mixed species biofilms. Compared to single species MBIC for each individual bacterium in the mixed biofilm, the mixed species MBIC unexpectedly required lower or equal concentrations of antimicrobials compared to the MBICs obtained for each bacterium in the single species biofilms. One exception was observed for *L. monocytogenes 568* and *S. baltica* treated with trans-cinnamaldehyde, where the *L. monocytogenes 568* dominated mixed biofilm had a higher MBIC than those found in the single species biofilms made by each bacterium. This result suggests a synergistic effect occurring in the mixed biofilm when treated with trans-cinnamaldehyde while other combinations with lower MBICs than single species biofilm suggested detrimental effects on survival of bacteria co-occurring in forming mixed species biofilms.

The doses required for eradication of bacteria in mixed biofilm compared to the individual bacteria in single species biofilm displayed similar trends. The mixture of *L. monocytogenes 568* and *S.* Typhimurium was eradicated by all four antimicrobial compounds, however, MBECs were lower in the mixed biofilm compared to the values for the single species biofilms. One exception was when the mixture was treated with

lemongrass EO, the mixed biofilm required a higher MBEC compared to single species biofilm. Lemongrass EO was proven throughout this study to be a poor anti-biofilm treatment and the mixed biofilm showed an increase resistance to the natural antimicrobial compound while in other cases the mixed bacterial biofilm increased susceptibility.

The established mixed biofilm made by *L. monocytogenes 568* and *P. fluorescens* exhibited higher MBECs following treatment with each of the four natural antimicrobial compounds as compared to MBECs obtained for each bacterium in the single species biofilms showing that for this combination of species, forming a mixed biofilm increased resistance to the natural antimicrobials. Carvacrol was the only compound to eradicate the *L. monocytogenes 568* and *S. baltica* mixed biofilm and was also the only natural compound where it was observed that a lower or equal MBEC was obtained for the mixed *L. monocytogenes 568* and *S. baltica* biofilm than for its bacterial components in their respective single species biofilms. These results showed that when the biofilms of *L. monocytogenes 568* and *S. baltica* were mixed and treated with carvacrol, a detrimental effect on growth and survival occurred compared to single species

The effect of the natural antimicrobials varied among compounds and mixed species biofilms; some cause detrimental effects while other combinations are beneficial compared to forming single species biofilm. Carpentier and Chassing (2004) studied mixed biofilms of *L. monocytogenes* with 29 other bacteria, their findings were also variable and showed that while some bacteria decreased the growth of *L. monocytogenes*, such as *P. fluorescens*, others increased its growth and others had no effect at all. *S. baltica* and *P. fluorescens* have been previously found to have opposite effects on the

growth of *L. monocytogenes* in mixed biofilms (Daneshvar Alavi and Truelstrup Hansen 2013). While *S. baltica* significantly increased the specific growth rate and prolonged the lag phase of *L. monocytogenes 568*, *P. fluorescens* decreased the growth rate and shortened its lag phase. *L. monocytogenes 568* also reduced the growth rate of *P. fluorescens* significantly but not *S. baltica.* When detrimental effects are observed in the mixed species biofilm compared to the single species, possible causes could be due to competition for nutrients preventing the bacteria of attaining optimal growth. Also, it is possible that the EPS formed by one of the components in the mixed biofilm may condition the attachment surface, preventing the second component in the mixture to form EPS (Leriche and Carpentier 2000).

There is limited knowledge on the effect of natural antimicrobial compounds on mixed species biofilms formed by *L. monocytogenes* with *S.* Typhimurium, *P. fluorescens* or *S. baltica,* although previous studies based on mixed biofilm disinfection assays have found both contradictory and similar findings. Van der Veen and Abee (2011) investigated the formation over 48 hours of single and mixed species biofilm of *L. monocytogenes* and *L. plantarum* as well as their resistance to disinfectants BAC and PAA at 20 °C. Their results indicated that mixed species biofilms were more resistant than single species. Similar to the findings of the eradication of *L. monocytogenes 568* and *P. fluorescens*, Giaouris et al. (2013) found that when mixed biofilms of *L. monocytogenes* and *P. putida* were formed, *L. monocytogenes* increased *P. putida's* resistance to BAC disinfection treatments compared to single species biofilms, but *P. putida* did not significantly influence the resistance of *L. monocytogenes* to BAC.

In agreement to findings of the current study, biofilm formation of *L. monocytogenes Scott A* and *L. monocytogenes FM876* was evaluated as single species and mixed species biofilm with *P. fragi* and *S. xylosus*. The *L. monocytogenes* strains had greater adherence in monoculture than in mixed-culture biofilms (Norwood and Gilmour 2001). Possible explanations for the results were the competition for nutrients, negative effects on the growth of *L. monocytogenes* when *Pseudomonas* and *Staphylococcus* were present in biofilms and the production of antagonistic compounds by *S. xylosus*. Kostaki et al. (2012) found a mutualism in a biofilm formed by *L. monocytogenes* and *S. enterica* and stated that interspecies interactions did not have an effect on their biofilm forming ability. However, the effect observed between mixed and single species conditions was found to be dependent on both species and the disinfectant applied. Both *L. monocytogenes* and *S. enterica* became less resistant when treated with a hydrogen peroxide and PAA mixture as a mixed species than as a single species biofilm while no differences were observed when treated with BAC or NaClO. The effect of *Cinnamomum cassia* EO and cinnamaldehyde were tested against a mixed biofilm consisting of *L. monocytogenes* and enteropathogenic *E. coli*. Results showed that the treatments affected both species equally, however, mixed species biofilms were more sensitive to treatments than single species biofilms (de Oliveira et al. 2012).

The variation in these findings further proves that biofilms are very diverse and unique and their resistance, attachment and growth is dependent on many environmental conditions. These findings also show the importance of studying mixed species biofilms since they exist in food processing environments and can contribute to lower or higher bacterial resistance especially as they mature. Further knowledge on the effects of each

species' EPS forming abilities in mixed species biofilms as well as the anti-biofilm action and mechanisms of the natural antimicrobial compounds should be explored to help explain the interactions among bacteria in mixed biofilms and their resistance to natural antimicrobial compounds. Several studies explore mixed species biofilms but do not necessarily compare their results to the single species biofilm of each bacterium in the mixed biofilm using the same experimental parameters preventing true comparisons and conclusions about resistance to be made.

5.5 Determination of Bacterial Species Proportions in Mixed Species Biofilm Inhibition (MBIC) and Eradication (MBEC) Assays

The proportions of each bacterial species varied by treatment, maturity and composition of the mixed biofilm. The proportions of the bacteria in a mixed species biofilm represent the final population of each bacterium and are a result of the competition between the bacteria for available nutrients and surface to colonize (Leriche and Carpentier 2000). To confirm that the composition of the mixed biofilm is dependent on treatment dosage, the proportions have also been observed to change when treated with sub-inhibitory concentrations. It has been observed that the bacteria do not grow equally in the mixed biofilms as well as some bacterial mixes caused detrimental effects to each other's survival against treatment while other bacterial mixes impacted a beneficial effect or made no difference compared to the single species biofilms. In some cases (e.g., carvacrol treated *L. monocytogenes 568* and *S.* Typhimurium*, L. monocytogenes 568* and *P. fluorescens*) although the biofilms became dominated by one species, the mixed biofilm population was still less resistant to the antimicrobial compound compared to the dominant bacterium's single species biofilm showing that the presence of the partner bacteria had a negative effect.

When comparing MBICs for all four natural antimicrobial compounds for the *L. monocytogenes 568* and *S.* Typhimurium mixed biofilm, higher resistance was seen when treated with thymol and carvacrol which were both dominated by *L. monocytogenes 568*. Similarly when comparing MBICs of *L. monocytogenes 568* and *S. baltica* mixed biofilm for all four compounds, higher resistance was seen when treated with carvacrol which interestingly is the only case where *L. monocytogenes 568* outcompeted *S. baltica*. These findings suggest that elevated *L. monocytogenes 568* presence contributes to biofilm resistance. Kostaki et al. (2012) found that in general *L. monocytogenes* seems to be more resistant than *S. enterica* to BAC, PAA, NaClO and hydrogen peroxide independent of growth conditions. *L. monocytogenes* is also known to be able to grow, adapt and survive in a broader range of environmental conditions. Although a mutualism was observed in the *L. monocytogenes* and *S. enterica* mixed biofilm, following disinfection treatments, the biofilm was mainly composed of *L. monocytogenes* ranging from 67.6 to 97.1% depending on the disinfectant. These results agree with the present findings, where *L. monocytogenes 568* dominated the *L. monocytogenes 568* and *S.* Typhimurium mixed biofilm when left untreated and for thymol, carvacrol and trans-cinnamaldehyde treatments but was outcompeted by *S.* Typhimurium when treated with lemongrass EO.

L. monocytogenes 568 and *S.* Typhimurium was the only mixture with MBECs for all four natural antimicrobial compounds suggesting it was the weakest biofilm mixture for this assay. The development stage played a role in the final population, although initially outcompeted, *S.* Typhimurium cells were able to survive throughout the incubation period and regain numbers as the biofilm matured. This could be due to an

ageing *L. monocytogenes 568* population or *S.* Typhimurium may have higher survival abilities when nutrients are depleting.

Chorianopoulos et al. (2008) formed a mixed species biofilm for 5 days at 16 °C consisting of *St. simulans*, *L. fermentum*, *P. putida, S. enterica* and *L. monocytogenes* and revealed that is was mainly composed of *P. putida* cells. *S. enterica* and *L. monocytogenes* together only contributed to 2.2% of the biofilm while *St. simulans* and *L. fermentum* were not found in the final biofilm population. *S. enterica* has been previously reported to form 1.5 log less biofilm in a mixed biofilm with *St. simulans*, *L. fermentum*, *P. putida* and *L. monocytogenes* then as a monoculture while *L. monocytogenes* did not differ between mixed and single species biofilms. The study also found that the essential oil *Satureja thymbra* at 1% (v/v), which contains carvacrol and thymol, was an effective anti-biofilm agent against single and mixed species biofilm. Van der Veen and Abee (2011) found that an untreated mixed biofilm of *L. monocytogenes* and *L. plantarum* was dominated by *L. monocytogenes,* however, the addition of manganese sulfate and/or glucose altered the composition by decreasing the *L. monocytogenes* population and increasing the *L. plantarum* population.

The bacterial proportions of the established *L. monocytogenes 568* and *P. fluorescens* mixed biofilm were also affected by maturity; *P. fluorescens* was the dominant bacteria in the untreated control but decreased in dominance as the biofilm matured. *P. fluorescens* is known to be a fast and proliferous bacteria with strong biofilm forming abilities, a reduction in dominance may be due to the bacteria ageing faster than *L. monocytogenes 568. L. monocytogenes 568* may also be more resilient and can survive when nutrients and oxygen become low after long incubation periods. The proportions

showed high dependence on the treatment, when the *L. monocytogenes 568* and *P. fluorescens* mixed biofilm was treated with the trans-cinnamaldehyde MBEC, *P. fluorescens* became highly susceptible allowing *L. monocytogenes 568* to overtake the bacterial community. This susceptibility to trans-cinnamaldehyde was also seen in the MBEC assays of *P. fluorescens* as a single species biofilm. Giaouris et al. (2013) found that mixed biofilms consisting of *L. monocytogenes* and *P. putida* was composed of 90% *P. putida* after 2 days incubation and when treated with BAC it mainly killed *L. monocytogenes* cells. *Pseudomonads* are known for their ability to form strong biofilm consisting of EPS and generally have high intrinsic resistance compared to other Gram-negative bacteria.

For the mixed biofilm of *L. monocytogenes 568* and *S. baltica*, the determination of proportions were limited to untreated controls. For the untreated control the results proved once again that maturity of the mixed biofilm impacted the final population of bacteria in the mixed species biofilm. While *S. baltica,* dominated the biofilm at 3 days, it was outcompeted by *L. monocytogenes 568* after 5 days' incubation. This would suggest that most of the *S. baltica* cells have died after 5 days or have weakened and were outcompeted by *L. monocytogenes 568* since it was present at such a high percentage.

The presence of *P. fluorescens* and *S. baltica* was previously observed to outcompete and negatively affect the proportion of *L. monocytogenes 568* in dual species biofilm, however, when each mixture was exposed to a 21-day desiccation period the surviving population of *L. monocytogenes 568* cells were greater than both Gram-negative spoilage bacteria (Daneshvar Alavi and Truelstrup Hansen 2013). The results in the present study are comparable, when left untreated, the spoilage bacteria (*P.*

fluorescens and *S. baltica*) dominated *L. monocytogenes 568* in the mixed species biofilm and lowered resistance compared to single species biofilm. However, for some treatments (e.g., MBIC of carvacrol and thymol for *L. monocytogenes 568* and *P. fluorescens*) the spoilage bacteria showed greater susceptibility allowing *L. monocytogenes 568* to dominate.

The results highlight the importance of studying the interactions between the bacteria in mixed biofilm as well as their proportions to fully understand which bacterial component is present at higher levels and how to effectively remove and kill the biofilm. If only one of the species is inhibited then the other species will thrive in the environment without any competition. Further studies on the proportions of mixed biofilm such as starting at unequal bacterial concentrations and submitting these biofilms to inhibitory and sub-inhibitory concentrations of treatment would help the understanding of the interactions in the mixed biofilm. This is important because in a food processing environment it is not likely each bacterial species will be present in mixed biofilms in a 50/50 ratio.

There is a lack of previous literature studying mixed biofilms at different development stages but since the proportions of each bacterial component changed, in some cases drastically, further exploration should be conducted to ensure proper dosages of antimicrobials are applied to cover all maturities and all mixtures. Mixing more than two of the bacteria would also be beneficial to get dosages that cover a broader range of mixtures, although it's been proven that each mixture for each natural antimicrobial compound yields unique results.

5.6 Scanning Electron Microscopy (SEM) of Single and Mixed Species Biofilms.

SEM provides spatial images at high magnifications to allow observations of where the single bacteria are located and interact within the biofilm (Alhede et al. 2012). For listerial biofilms treated with MBICs determined by the CV staining assay, a reduction of biofilm following all four treatments compared to the positive control was observed. The biofilm incubated for 6 days at 15 °C showed much greater growth than the 3 day biofilm at 15 °C for *L. monocytogenes 568* which agrees with the chosen experimental parameters for successful CV staining. There were no signs of EPS or any additional masses for the positive control nor the treatments for the MBIC of *L. monocytogenes 568* suggesting that the bacteria lacks in ability to form EPS after 6 days incubation at 15 °C. *L. monocytogenes* biofilm that were pre-formed for 6 days at 15 °C then treated with MBECs had a higher amount of visible biofilm cells and some EPS in the untreated positive control. These findings agree with quantitative data; MBECs were higher or equal to MBICs which now can be suggested to be due to an increase in biofilm cells.

Daneshvar Alavi and Truelstrup Hansen (2013) observed *L. monocytogenes 568* under SEM and found that the biofilm consisted of microcolonies spread over the surface area with intermittent rod-shaped single cells which appeared to be attached to the surface by fibril-like structures; however large masses of EPS were absent. Rieu et al. (2008) found that the *L. monocytogenes* biofilms in pure culture were flat, homogenous and composed of small rods as well. Similar results were found in a study by Hingston et al. (2013) when *L. monocytogenes 568* was desiccated. SEM micrographs revealed a honeycomb like structure with a single layer of biofilm spread across the surface with

multiple layers of aggregated cells. Fibrils were also observed connecting cells together and onto the surface material, leading the authors to suggest that these connective fibrils were remnants of EPS.

For the mixed species biofilm of *L. monocytogenes 568* and *S.* Typhimurium, a reduction of biomass was observed for all four natural antimicrobial treatments compared to the positive control when treated with MBICs and MBECs. For the established biofilms treated with the MBECs, the population was reduced for each antimicrobial, but large masses of EPS were still observed in the treated mixed biofilm except for the carvacrol treatment which reduced both components. This follows quantitative data that the natural compounds can eradicate viable bacteria but are unable to remove the biomass. The untreated control for the MBEC determination of *L. monocytogenes 568* and *S.* Typhimurium was much more populated compared to the untreated control for the MBIC determination. This is due to the fact that the MBEC untreated control was incubated for 2 additional days after the natural compounds were added to the treatment wells.

Upadhyay et al. (2013) observed *L. monocytogenes* biofilms treated with carvacrol and thymol by confocal laser scanning microscopy and revealed that when treated, the biofilm consisted of mainly dead cells and breaks in the biofilm were observed while the untreated control consisted of a dense layer of live cells. The *L. monocytogenes* biofilm treated with trans-cinnamaldehyde harboured a mixture of live and dead cells. In agreement with present observations, these results indicate that although the cells were dead, they were still visible and present; meaning the compounds did not remove biomass.

The SEM imagery revealed that all four antimicrobial compounds at their MBIC, reduced the *L. monocytogenes 568* and *P. fluorescens* population and EPS structures but some EPS remnants surrounding the rods were still observable. The MBEC treated mixed biofilm of *L. monocytogenes 568* and *P. fluorescens* showed a reduction in biofilm population for all treatments which was unexpected since thymol was the only treatment to eradicate the biofilm at a level of 95% ± 5%. This could be due to poor attachment, eradication levels close to 95% ± 5%, high cell viability and lack of biomass. An increase in visible biological material surrounding cells was seen in established biofilms compared to biofilms grown in the presence of the treatment. This proves once again that established biofilm yeild larger amounts of EPS. Previous studies have observed how single cells in a *P. fluorescens* biofilm were connected by an amorphous mass assumed to be EPS (Daneshvar Alavi and Truelstrup Hansen 2013). The study also formed mixed biofilm with *L. monocytogenes 568* and *P. fluorescens* and similarly reported that the mixed biofilm resembled the single species biofilm of *P. fluorescens* only due to its dominance in the biofilm (>99% of the population).

SEM visualization of the mixed *L. monocytogenes 568* and *S. baltica* biofilms treated with the MBICs showed a reduction of population for carvacrol, trans-cinamaldehyde and lemongrass EO treated biofilm but not when treated with the MBIC of thymol. The thymol treated mixed biofilm appeared have an increase in large masses of biological material as well as a slightly increased bacterial population. The MBICs determined were for inhibition of viable cells and not removal of biofilm, although the population does not appear to have reduced, the number of viable cells may be less than the positive control. The mixed biofilm of *L. monocytogenes 568* and *S. baltica* treated

with the MBECs for all four compounds had SEM results that were well matched to quantitative data. The positive control for the *L. monocytogenes 568* and *S. baltica* mixed biofilm had the most observable biofilm, with a thick spread of cells covered in assumed EPS compared to other mixed biofilm. As seen with other mixed biofilm, the MBEC assays showed much greater biofilm and EPS presence than MBIC assays. Daneshvar Alavi and Truelstrup Hansen (2013) also formed mixed species biofilms of *L. monocytogenes 568* and *S. baltica* which resembled the *S. baltica* single species biofilm and consisted of a thick multicellular layer covering the surface embedded in unidentified biological material.

Similarly to present observations, in a mixed biofilm of *L. monocytogenes* and *St. aureus*, larger, elongated *L. monocytogenes* rods were observed and found within microcolonies of *St. aureus,* which may be due to stressed cells when nutrients diminish (Rieu et al. 2008). Carpentier and Chassing (2004) showed by epifluorescence microscopy that the spatial arrangement of *L. monocytogenes* cells within a biofilm changed depending on the second component. When mixed with *Kocuria varians, L. monocytogenes* cells gathered around the microcolonies while when mixed with *Comamonas testosteroni*, *L. monocytogenes* cells formed its own microcolonies. It appears in this study that the species gather together to form microcolonies however, no conclusions can be made, since all the bacteria tested are rod shaped and could compose mainly of one species. A fluorescent Gram stain must be used to distinguish the species.

The visualization of biofilm by SEM supported the quantitative data from staining assays. In many cases it was observed that the natural compounds were able to remove bacterial rods but additional biomass remained. Further testing to be done would include fluorescent microscopy with stains distinguishing viable and dead cells (Kadam et al. 2013). Further explorations in the composition of EPS and how to effectively remove it could be useful for control strategies for example Kadam et al. (2013) added DNase I to confirm the presence of eDNA in *L. monocytogenes* biofilm EPS. Unfortunately in mixed biofilm, the proportions of each bacteria using SEM cannot be determined effectively since all tested bacteria were rod-shaped. Future work could include fluorescent Gram stains to be evaluated using fluorescence dyes and epifluorescence microscopy to differentiate between bacteria (Hannig et al. 2010; Daneshvar Alavi and Truelstrup Hansen 2013).

CHAPTER 6 CONCLUSION

6.1 Project Summary

The antimicrobial and anti-biofilm properties of thymol, carvacrol, trans-cinnamaldehyde and lemongrass EO were determined on single and mixed species biofilm of *L. monocytogenes, S.* Typhimurium*, P. fluorescens, S. baltica* and *St. aureus*. The efficacy of the natural antimicrobial compounds on planktonic growth, mature biofilms and the removal of EPS was also determined. In addition, the proportions of the bacteria in the final population of mixed species biofilm were also revealed.

The natural antimicrobial compounds did not work equally well for each bacterium; throughout the study it has been discovered that the effect of the dosage of each antimicrobial compound is dependent on the bacteria, the compound and the maturity of the biofilm. All four natural compounds were effective as antimicrobials, as they were able to inhibit planktonic bacterial growth of all the tested bacterial species at relatively low concentrations. The four compounds were also effective in the eradication of planktonic cells concluding that thymol, carvacrol, trans-cinnamaldehyde and lemongrass EO are effective antimicrobials against planktonic pathogenic and spoilage bacteria. In all cases a higher concentration was required to eradicate the planktonic cells than to inhibit the growth showing that it is important to use a dosage that is able to eradicate the bacteria, so the cells do not persist in the food processing environment.

The four natural antimicrobial compounds were effective in the inhibition of biofilm for all the tested bacterial species in terms of inhibition of metabolic activity and biomass production (EPS). In all cases, a higher dosage was required to inhibit biofilm metabolic activity than to inhibit the formation of biomass. Biofilm proved to have an

increased resistance to the natural antimicrobial compounds compared to planktonic cells. The increased resistance in biofilms have been linked to the formation of EPS allowing for the target microorganism to be protected from the treatments. Thymol, carvacrol, trans-cinnamaldehyde and lemongrass EO prevented biofilm formation which is important in terms of selecting an appropriate disinfectant since it is more ideal to prevent biofilm formation in food processing environments than deal with the removal of established biofilms.

The four natural antimicrobial compounds were effective anti-biofilm agents but the efficacy was dependent on the maturity of the biofilm as well as the compound and bacterial species. In general, the resistance to all four natural antimicrobials increased as the biofilms matured. Biofilm that have been pre-formed also increased in resistance compared to biofilm forming in the presence of the natural compounds. The natural antimicrobial compounds were able to effectively kill pre-formed biofilm but did not necessarily remove or disperse it. The natural antimicrobial compounds have proven to effectively inhibit EPS production however removal of EPS only occurred in some Gram-negative bacteria.

Thymol, carvacrol, trans-cinnamaldehyde and lemongrass EO were also able to inhibit the formation of mixed dual species biofilms, which consisted of *L. monocytogenes* together with *S.* Typhimurium*, P. fluorescens* or *S. baltica*. The eradication of mixed species biofilm was not as successful, it required the highest tested concentrations or eradication did not occur suggesting an increased resistance to the antimicrobials in the established mixed biofilms. The concentrations required for eradication were dependent on the bacterial mixture and the antimicrobial compound. For

the inhibition of mixed species biofilms in most cases a higher concentration was required to inhibit single species biofilm than the mixed species, suggesting a detrimental effect to the resistance of the bacteria in the mixed biofilms. For the eradication of mixed species biofilm, the effect varied among mixed biofilms and natural antimicrobial compound, where in some cases the mixture deemed beneficial for the bacteria in terms of increased resistance to the natural antimicrobials while in other cases it lowered bacterial resistance.

The proportions of each bacterium in the mixed species biofilms were investigated and it was observed that each species does not grow equally well in a mixed species biofilm. The proportions were dependent on the bacterial species, the natural antimicrobial compound and the concentration of compound and the biofilm's development stage. SEM micrographs supported the quantitative data showing visible reductions of biofilm and EPS following effective anti-biofilm treatments and the presence of both in cases where the natural antimicrobial compounds were not effective.

This study successfully proved that the natural compounds thymol, carvacrol, trans-cinnamaldehyde and lemongrass EO are effective antimicrobials and anti-biofilm agents against *L. monocytogenes, S.* Typhimurium*, P. fluorescens , S. baltica* and *St. aureus*. The required treatment concentration was dependent on a number of factors and varied among species and natural compound. At the correct dosage, these natural antimicrobial compounds were shown to inhibit a wide range of microorganisms in several different growth scenarios and could be used as a natural alternative to control strategies in food processing environments. Although minimal inhibitory concentrations were determined, ideally to cover all situations such as mature, mixed biofilms and

resistant bacteria, the use of a higher concentration able to eradicate the biofilm regardless these factors would be recommended. Thymol and carvacrol were the most versatile and effective treatments although trans-cinnamaldehyde and lemongrass EO were very effective antimicrobials and biofilm inhibitors. Future work to establish concentrations for each natural antimicrobial compound strong enough to control mature mixed biofilms would be beneficial.

6.2 Future Directions

1. Understanding the composition and structure of the EPS matrix formed in single and mixed species biofilms in order to improve disinfection and removal strategies by directly targeting the EPS.

2. Mixing the natural antimicrobial compounds together or with synthetic compounds in order to improve efficacy.

3. Mixing all bacterial species from this study and observing their interactions could give further insight on the bacterial dynamics in mixed biofilms and the natural antimicrobial compound's effect on these complex mixtures.

4. The bacteria in mixed biofilm will most likely not be maintaining the starting ratio of 1:1, and future work could include using different starting proportions of each bacteria. The effect of the natural antimicrobial compounds on unequally mixed biofilms could also be examined.

5. Investigating the effect of the natural antimicrobial compounds on several strains of *S.* Typhimurium*, P. fluorescens, S. baltica* and *St. aureus*, since *L. monocytogenes* strains

showed differences in required inhibition and eradication concentrations for planktonic and biofilm cells.

6. Applying the natural antimicrobial compounds at different time intervals to determine how quickly the antimicrobials achieve the significant inhibition or eradication.

7. Testing a broader range of concentrations for the antimicrobial treatments that were unable to eliminate biofilm and for those where the lowest tested concentration caused inhibition and eradication to accurately determine miminal antimicrobial concentrations.

8. The effect of the natural antimicrobial compounds on single and mixed species biofilm should be tested on biofilm formed on different materials such as the food industry relevant stainless steel.

9. The use of viability staining and Gram-staining for fluorescence microscopy would enable a distinction between dead/alive and Gram-negative and Gram-positive bacteria in single and mixed species biofilms.

10. Making useful disinfection products from the natural antimicrobial compounds to be applied to food processing environments, which could include sensory tests in terms of residues leading to tastes and smells and making a cost efficient product.

11. Develop another method to evaluate mixed species proportions so that the biofilm population does not drop below the detection limit and proportions of bacteria in all the mixed biofilms can be determined.

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