The Use of Selected Phytochemicals to Control *Listeria monocytogenes*; Practical Applications in Food Safety

by

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ABSTRACT

Listeria monocytogenes is an important foodborne pathogen found in minimally processed foods, such as dairy products. To improve detection methods, phytochemicals were screened against *L. monocytogenes* and *L. innocua*. Efflux pump inhibitors promoted the growth for *L. monocytogenes* strain (FSL-J1-031) over *L. innocua* strain (2007-663). When tested against multiple strains of *Listeria* there was no clear differential effect observed. Aiming to control *L. monocytogenes*, biofilm growth kinetics of 2% Milk Fat pasteurized milk at 4°C was investigated, with a mature biofilm-forming at 74 h. Preformed biofilms were exposed to 20 phytochemicals *in vitro* with carvacrol, thymol, eugenol, coriander essential oil, and tea tree essential oils removing between 89% and 97% of the biomass. When tested in dispensing pumps, phytochemicals reduced biomass by 17% (thymol) and 32% (thymol) with mechanical force. Further research is needed to identify natural antimicrobial agents and techniques that are more effective for controlling *L. monocytogenes*.

LIST OF ABBREVIATION USED

ANOVA: Analysis of variance

BC: Benzalkonium chloride

BHI: Brain-heart infusion broth

CCCP: Carbonyl cyanide 3-chloro phenyl hydrazine

CFIA: Canadian Food Inspection Agency

CFU: Colony forming unit

CV: Crystal violet

DMSO: Dimethyl sulfoxide

EO: Essential oils

MATE: Multidrug and toxic compound extrusion

M.F: Milk fat

MFS: Major facilitator superfamily

MIC: Minimum inhibitory concentration

NMP: 1-(1-naphtylmethyl) piperazine

OD: Optical density

PBS: Phosphate-buffered saline

QAC: Quaternary ammonium compounds

RND: Resistance-nodulation-cell division

RTE: Ready to eat

SMR: Small multidrug resistance

TSA: Trypticase soy sgar

UVM1: University of Vermont medium

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CHAPTER 1 INTRODUCTION

Listeria monocytogenes is a facultative anaerobic non-spore forming Gram-positive bacterial foodborne pathogen (Chakraborty, 1999). *Listeria monocytogenes* cells are able to survive and replicate in environments considered adverse to other bacteria (Valderrama and Cutter, 2013). These adverse environmental conditions are commonly used for food preservation such as low pH, low temperature, and high salt concentration (Valderrama and Cutter, 2013). *Listeria monocytogenes* is the cause of listeriosis, which is characterized by high mortality rates of up to 30%, with clinical characteristics of meningitis, septicemia, gastroenteritis, abortions and perinatal infections (Braun and Cossart, 2000). The most susceptible individuals are those with decreased immunity such as neonates, fetuses in pregnant women, the elderly, immunosuppressed transplant recipients as well as others with impaired cell-mediated immunity (Doganay, 2003). Strains of *L. monocytogenes* are classified into 13 serotypes, where serotypes 1/2a, 1/2b, and 4b are responsible for most of listeriosis cases, with the majority of invasive outbreaks are due to 4b serotype strains (Laksanalamai et al, 2012). Globally, in 2010, 23,000 individuals were diagnosed with listeriosis, with 5,500 cases resulting in death (Thomas et al, 2015). *L. monocytogenes* infections are in fact the leading cause of death due to foodborne illnesses (Wallace et al, 2017). The severity of the disease makes it one of the most important foodborne pathogens both economically and with regard to public health (Møretrø et al, 2017). In Canada, the most recent outbreak of foodborne listeriosis occurred in 2008, where resulted in 57 cases were reported, 24 of which resulted in death. The associated economic burden of this outbreak was estimated to be nearly 242 million Canadian dollars (Thomas et al, 2015). Although *L. monocytogenes* can withstand mild heat treatments in foods (<60°C) it is relative sensitive to higher temperatures (71°C), therefore foods consumed without further heat treatment (cooking),

known as ready-to-eat (RTE) foods, such as milk and cold-cut meat products, are the main source for listeriosis outbreaks (Møretrø et al, 2017).

In the context of food safety and food microbiology, persistence can be defined as the repeated isolation of a foodborne pathogen of a specific molecular subtype from the same food processing environment despite the frequent and correct application of cleaning and disinfection procedures (Overney et al, 2017). One way for *L. monocytogenes* to persist in an environment is to form biofilms or colonize pre-existing biofilms (Røder et al, 2015). Bacterial biofilms can cause problems in many areas of the food industry as they enable bacterial pathogens to better withstand standard cleaning procedures (Røder et al, 2015). Biofilms composed of multiple bacterial species may become more resistant towards antibacterial agents including antimicrobials, surfactants and detergents such as the commonly used quaternary ammonium compound (QAC), benzalkonium chloride (BC) (Røder et al, 2015). There are many mechanisms that contribute to the increased antimicrobial resistance of biofilms such as weak diffusion of antibiotics and detergents through the biofilm exopolysaccharide matrix, as well as phenotypic changes of the cells forming the biofilm. However, the expression of molecular efflux pumps has been recently found to contribute to the increased resistance found in biofilms (Liu et al, 2017). Efflux is a key mechanism of cellular response to varied environments and allows the microorganisms to regulate their internal environment by removing toxic substances such as antibiotics and BC, a commonly used disinfectant in the food industry (Liu et al, 2017). Efflux pumps have also been found to be important for the secretion of the exopolysaccharide matrix of biofilms (Liu et al, 2017). As efflux has multiple roles in biofilm formation, inhibiting the function of efflux pumps may inhibit biofilm formation leading to a reduced persistence of *L. monocytogenes* and, consequently, a reduced occurrence of listeriosis outbreaks.

1.1 Thesis Hypotheses

1. Phytochemicals found in essential oils have a differential effect on *L. monocytogenes* and *L. innocua* due to species-specific inhibitory mechanisms, with a specific focus on efflux pumps that can be utilized to improve the detection methods of *L. monocytogenes*.

2. Specific phytochemicals will sufficiently remove *L. monocytogenes* and multispecies biofilms with practical applications in commercial milk dispensing pumps.

1.2 Thesis Overall Objective

1. To identify a phytochemical or combination of phytochemicals that would not affect the growth of *L. monocytogenes* while inhibiting the growth of *L. innocua,* to improve the detection of *L. monocytogenes* in food products.
2. To use phytochemicals that inhibit the growth of *L. monocytogenes* to eradicate multi-species biofilms formed in milk dispensing units, which potentially would shelter *L. monocytogenes*.

1.3 Thesis Specific Objectives

1. To screen up to 20 phytochemicals for differential bactericidal effect on *L. monocytogenes* and *L. innocua.*

2. To investigate the ability of *L. monocytogenes* to colonize preformed multispecies biofilms and assess the ability of identified phytochemicals to disrupt these multispecies biofilms.

3. To establish practical cleaning procedures to remove biofilms formed within industrial milk dispensing equipment.

CHAPTER 2 LITERATURE REVIEW

2.1 Outbreaks of Listeriosis

Human listeriosis is relatively rare despite our likely frequent encounters with *L. monocytogenes* from a variety of sources including water, soil, vegetation, farm and rural environments, and urban environments (Gilmour et al, 2010). However, when outbreaks occur, there is high morbidity and mortality (20-40%) associated with invasive listeriosis infections (Allen et al, 2016). The majority of cases of listeriosis (99%) are foodborne with the most commonly implicated vehicles being RTE food products such as meat, dairy, seafood, and fresh produce that become contaminated with *L. monocytogenes* during production and/or processing (Gilmour et al, 2010; Allen et al, 2016). *Listeria monocytogenes* was first recognized as a foodborne pathogen in 1981 after an outbreak occurred in Nova Scotia, Canada linked to contaminated coleslaw (Allen et al, 2016). Since then, in the United States of America, *L. monocytogenes* causes over 1600 illness and 250 deaths, annually (Angelo et al, 2017). A recent multi-state outbreak occurred in whole apples that were utilized for caramel apples from October 2014 to February 2015 (Angelo et al, 2017). There were 35 outbreak-associated cases in 12 states, with 34 of the individuals being hospitalized and 7 deaths (20%). (Angelo et al, 2017). Prior to this event, whole apples had not been associated with food recalls outbreaks involving *L. monocytogenes* outbreaks (Angelo et al, 2017). This is an indication that new foods are being implicated in outbreaks of foodborne listeriosis. Another multi-state outbreak of foodborne listeriosis, the largest to date in the U.S.A., occurred in 2011 where there were 147 cases in 28 States were reported, with 143 hospitalizations and 33 deaths, with one death being a miscarriage (McCollum et al, 2013). The contaminated product was found to be cantaloupe from a single Colorado farm (McCollum et al, 2013). An additional multi-province outbreak of foodborne

listeriosis that occurred in Canada in 2008 that was associated with delicatessen meat (Currie et al, 2015). There were 57 cases of listeriosis and 24 deaths, which all occurred at a long-term care facility (Currie et al, 2015). This outbreak of foodborne listeriosis demonstrated the need to improved listeriosis surveillance, strict control of *L. monocytogenes* in food processing facilities associated with RTE food products as it was the largest invasive listeriosis in Canada to date and the first associated with RTE meats (Currie et al, 2015). Other food products associated with *L. monocytogenes* outbreaks are dairy products such as raw milk, pasteurized milk, and soft cheese all-seeing death rates between 12% and 30% (Jackson et al, 2018; Hanson et al, 2019; Costard et al, 2017). Many of the cases with pasteurized dairy products resulted due to a mixture of raw contaminated milk or contact with a surface contaminated with *L. monocytogenes* (Jackson et al, 2018).

2.2 Biofilms

A biofilm is a single species, or multi-species, a community of microorganisms that have formed a matrix consisting of exopolymeric substances and water that is firmly attached to a diverse range of surfaces (Gião and Keevil, 2012). Studies of single-species biofilms have shown distinct basic steps in biofilm formation, which include: (1) initial attachment to a surface, followed by (2) the formation of micro-colonies, and finally (3) maturation of the micro-colonies into a matrix-encased mature biofilm (Chang et al, 2012). Planktonic cells (free-floating organisms) undergo profound biological changes such as a change in gene expression during the transition to sessile life in the surface-attached community (Chang et al, 2012). Typically, *L. monocytogenes* can attach to common materials found in food processing environments such as glass, plastic, and stainless steel (Alonso et al, 2014). Biofilms are a microbial protective mode of living, enabling the microorganisms to survive adverse environmental conditions (Wassinger et

al, 2013). As biofilms allow *L. monocytogenes* to survive in difficult environments, persistent strains of *L. monocytogenes* have an increased chance of contaminating food products, leading to product recalls and potentially outbreaks of listeriosis (Fox et al, 2011; Brider et al, 2015). Biofilm development and formation vary among *L. monocytogenes* serotypes, lineages, and origins of isolation, as well as with varying and intrinsic and extrinsic factors (Kadam et al, 2013). It has been reported that *L. monocytogenes* serotypes 1/2b and 1/2a have a higher ability to form biofilms than serotype 4b (Kadam et al, 2013). Biofilms composed of multiple bacterial species have increased resistance towards antibiotics and detergents than single-species biofilms (Røder et al, 2015). In the case of *Listeria*, a significantly higher number of *L. monocytogenes* cells are known to attach to stainless steel surfaces when residing in mixed-species biofilms compared to its monoculture biofilms (Gandhi and Chikindas, 2007). Similarly, *L. monocytogenes* cells from a mixed-species biofilm are able to survive for longer periods of time (Gandhi and Chikindas, 2007). This is important in food environments as many microorganisms are found within foods and food processing facilities, which increases the possibility for mixed culture biofilms to develop (Gandhi and Chinkindas, 2007). The effective removal of biofilm typically requires substantial mechanical treatments; hence, there is an increasing interest is increasing in substances that prevent and remove biofilms (Szczepanski and Lipski, 2014).

2.3 Efflux Pumps

Molecular efflux pumps are protein complexes inserted in the cellular membrane that are responsible for changes in the structural changes in the cell membrane affecting its function (Romanova et al, 2007). Efflux pumps are able to extrude specific compounds out of the bacterial cell using proton motive force (Jiang et al, 2016; Romanova et al, 2007). Oxacillin, clindamycin and QAC resistance in *L. monocytogenes* is attributed to efflux pumps (Camargo et al, 2015;

Møretrø et al, 2017). Acriflavine, a toxic dye, is also extruded out of *Listeria* species by an efflux pump, allowing for members of this genus to be naturally resistant to the compound (Abreu et al, 2012). In the presence of reserpine, a known plant-derived efflux pump inhibitor, *L. monocytogenes* was found to be generally more sensitive to the effects of acriflavine than other *Listeria* spp. (Langille, 2015; Abreu et al, 2016). There are five families of drug efflux systems: major facilitator superfamily (MFS), resistance-nodulation-cell division family (RND), small multidrug resistance family (SMR), multidrug and toxic compound extrusion family (MATE) and ATP- binding cassette superfamily (Guérin et al, 2014). *Listeria monocytogenes* contains two efflux pumps both belonging to the MFS, the first being encoded by the *mdrL* gene, is responsible for the efflux of macrolides, cefotaxime, and heavy metals, while the second encoded by the *lde* gene, is responsible for the efflux of fluoroquinolone, acriflavine and ethidium bromide (Guérin et al, 2014). Recently, however, MATE efflux pumps were documented to be activated in the resistance of fluoroquinolone in *L. monocytogenes*, and this is unusual as MATE efflux pumps are not commonly found in Gram-positive bacteria (Guérin et al, 2014). An important characteristic of MATE efflux pumps is that they are able to recognize and expel acriflavine. By screening a transposon-insertion library of *L. monocytogenes* against acriflavine, Langille (2015) found that *L. monocytogenes strain 568* relies exclusively on a MATE efflux pump to expel acriflavine, rather than on its previously identified MFS efflux pumps that *L.innocua* use.

2.4 Phytochemicals

Phytochemicals or plant secondary metabolites are compounds that include essential oils (EOs) (Calo et al, 2015). EOs are volatile isoprenoids that plants produce for environmental interactions as either a protectant or attractant (Nguefack et al, 2004). EOs can be classified as

phytochemicals and have been found to possess antimicrobial properties and be effective in a variety of applications by decreasing the growth and survivability of microorganisms (Calo et al, 2015). EOs act as nonspecific antimicrobials by interfering with and destabilizing the operation of the phospholipid bilayer of the cell membrane, enzyme systems, and/or the genetic material of microorganisms (Abdollahzadeh et al, 2014). Chemically, EOs generally consist of terpene compounds, alcohols, acids, esters, epoxides, aldehydes, ketones, amines and sulfides (Calo et al, 2015). Therefore, the components of EOs can be divided into two major groups: i) terpene compounds and ii) compounds responsible for the aroma of EOs (Calo et al, 2015). Some of these compounds are trans-cinnamaldehyde, carvacrol, and thymol which have all have been shown to decrease *L. monocytogenes* motility and down-regulate the expression of many virulence genes (Upadhyay et al, 2012). EOs are currently being used in food systems as natural flavoring agents including thyme, oregano, and cinnamon oil, where the major phenolic compounds present are found to be thymol, ρ-cymol, carvacrol, cinnamaldehyde, and eugenol, respectively (Szczepanski and Lipski, 2014). Specifically, thymol, carvacrol, and eugenol, have shown significant antibiofilm activity against *L. monocytogenes* on stainless steel surfaces (Miladi et al, 2017; Sandasi et al, 2009). The antibiofilm mechanism by which these phenolic compounds were found to act was attributed to their ability to modify the activities of efflux pumps (Miladi et al, 2017; Miladi et al, 2016). Although EOs have been shown to inhibit biofilm formation, it has not been demonstrated that they can completely remove a preformed, mature multispecies biofilms (Szczepanski and Lipski, 2014). A combination of EOs that weaken *L. monocytogenes* and traditionally used disinfectants in food processing facilities, such as BC or bleach have been required thus far to completely remove mature biofilms (Szczepanski and Lipski, 2014).

There are a number of phytochemicals known to inhibit efflux pumps, causing microorganisms to become more susceptible to antimicrobial compounds. An effective efflux pump inhibitor decreases the dose of an antimicrobial agent required for bacterial inhibition (Abreu et al, 2012). Piperine has been found to reduce the MIC of mupirocin used to typically treat *Staphylococcus aureus* by inhibiting the MdeA efflux pump (Mirza et al, 2011). Ferruginol was also found to inhibit the NorA efflux pump found in *Staphylococcus aureus,* thereby increasing the bacterium's susceptibility to ethidium bromide (Smith et al, 2007). There are numerous reports of phytochemicals such as piperine, silybin and genistein having efflux pump inhibiting properties with similar results as mentioned above (Abreu et al, 2012).

2.5 Commercial Cleaning Procedures

Due to *L. monocytogenes* being ubiquitous in the environment, its control and eradication are important requirements within the food processing facilities in order to mitigate the occurrence of foodborne outbreaks of listeriosis. (Møretrø et al, 2017). The main route of contamination is thought to be through biofilm formation which allows the bacterium greater resistance to cleaning techniques. The most common disinfectants utilized in health care, agriculture, home, and the food industry are quaternary ammonium compounds (Gerba, 2015). Benzalkonium chloride (BC) is a commonly used quaternary ammonium compound, which typically contains a mixture of molecules with alkyl chain lengths of C12-C16 (Møretø et al, 2017). However, *L. monocytogenes* strains have shown increased tolerance to BC by varying degrees due to the presence of at least two genetic determinants *qac*H and *bcr*ABC, in the pathogen (Møretø et al, 2017). This increased resistance to BC has also been found to be the result of overexpression of other efflux pumps specific for other substrates different from BC (Ortiz et al, 2014). Therefore, utilizing sub-minimal inhibitory concentrations to disinfect

surfaces can create selective advantages for resistant strains of *L. monocytogenes*; thereby allowing them to persist (Ortiz et al, 2014).

2.6 Detection of *Listeria monocytogenes*

There is an increase in consumer demand for minimally processed RTE food products. Since these foods may allow for the growth of *L. monocytogenes* during their stated shelf life, reliable detection methods for *L. monocytogenes* are required (Engelhardt et al, 2016). Several culture-based detection methods have been developed which contain enrichment steps employing different selective media (Englehardt et al, 2016). In Canada, the detection of *L. monocytogenes* in food products is the responsibility of the Canadian Food Inspection Agency (CFIA), as well as of the Provincial food safety laboratories, where methods approved by Health Canada for the isolation and enumeration of *Listeria* from foods matrices (named MFHBP-30 and MFLP-74, respectively) are followed (Government of Canada, 2011). Method MFHPB-30 uses an enrichment broth known as UVM1 which contains selective inhibitors, the antibiotic nalidixic acid and acriflavine, in which *Listeria* spp. is naturally resistant. Nalidixic acid and acriflavine selectively inhibit the growth of microflora that are normally present in food (Carvalheira et al, 2010; Beumer et al, 1996), aiding in the isolation of *Listeria*. However, because this enrichment broth does not specifically discriminate between *Listeria* species, the non-pathogenic species such as *L. innocua* (which is often found in food processing environments and food products) is often co-isolated with *L. monocytogenes* (Carvalheira et al, 2010). This is a concern as the presence of certain strains of *L. innocua* can mask, or even inhibit the growth of *L. monocytogenes* mainly because *L. innocua* has a rapid growth rate which gives it an advantage over *L. monocytogenes*. (Carvalheira et al, 2010). This could lead to results that are considered false negatives as they will show no Colony Forming Units (CFUs) for *L. monocytogenes*,

therefore deeming a food product safe for consumption, when in reality it is not (Carvalheira et al ,2010).

2.7 Bovine milk as an important RTE food involved in listeriosis

Bovine milk is a nutritious food product consumed as a beverage or used as an ingredient in the production of a wide range of dairy and non-dairy products (Porcellato et al, 2018). Along with its nutritional aspects, raw milk contains a highly diverse bacterial population, which can lead to microbial contamination throughout the dairy chain (Porcellato et al, 2018). To ensure the safety of milk products, the dairy industry utilizes the technique known as pasteurization to kill pathogenic microorganisms, inactivate most (>95%) spoilage organisms and denature enzymes degradative excreted by microorganisms (Melini et al, 2017). Milk that has undergone correct pasteurization treatment is, therefore, unlikely to cause disease; however, in cases where inadequate heat treatment was applied or where recontamination events occur after pasteurization, harmful pathogens such as *L. monocytogenes* may be present in the milk (Melini et al, 2017). Additionally, compared to raw milk, the microflora associated with pasteurized milk has been found to have significantly greater proportions of bacteria belonging to the *Lactobacillales, Pseudomonadales, Clostridiales* and *Bacillales* (Porcellato et al, 2018). A number of bacterial species within these are known for their ability to adhere to the many surfaces found in the dairy system, including stainless steel, plastics, rubber, and polypropylene, which can lead to multi-species biofilm formation (Yuan et al, 2019). In fact, extensive biofilm formation has been found on various milk contact surfaces, such as storage tanks, transportation tankers, milk pipelines and heat exchangers (Yuan et al, 2019). As previously mentioned, these biofilms can be a source of contamination and can pose a serious health risk. It is therefore important to implement efficient cleaning procedures to decrease the risk of mature biofilm

formation. In the case of cleaning dairy processing lines, cleaning-in-place procedures are utilized to establish control over biofilms (Shi and Zhu, 2009). These procedures use chemical agents such as detergents, biological means like enzymes, as well as including physical methods such as mechanical brushing to remove preformed biofilms; however often is the case that microorganisms remain and persists within the system (Shi and Zhu, 2009).

The first outbreak of foodborne listeriosis in milk products occurred in U.S.A in 1983 with 49 cases being reported (Fleming et al, 1985). Forty-two of the cases occurred in immunosuppressed adults and 7 in mother-infant pairs (Fleming et al, 1985). The source of *L. monocytogenes* was within the whole or 2% pasteurized milk that was present in large numbers in the raw milk and was able to survive the pasteurization period (Fleming et al, 1985).

CHAPTER 3 MATERIALS AND METHODS

3.1 Bacterial Strains and Culture Conditions

Bacterial strains that were used throughout this study were stored in beads in cryovials at -80°C for long term storage (Microbank™, Pro-lab Diagnostic, Richmond, Ontario, Canada). For short term storage (two weeks), cultures were grown on trypticase soy agar plates (TSA) and then stored at 4°C. All bacterial cultures were initially grown by removing a single bead from a -80°C stock cryovial and streaking onto a plate of TSA agar (Difco). All TSA agar plates were incubated at 30°C for 24 hours (Isotemp™ incubator, Fisher Scientific, Ottawa, Ontario, Canada). Agar plates were stored at 4°C for two weeks for the use of picking single colonies to provide cultures for the downstream experiments. The list of bacterial strains used throughout this study is given in Table 1.

Table 1: Details of the *Listeria* strains used in this investigation.

Listeria Strain ID	Species	Sero type	Source	Isolated From	Legend
1. St. Hy-2003-0034-0001	*L. monocytogenes*	1/2b	CFIA Dartmouth Lab	Unpasteurized cheese	L. mono (1)
2. DAR-FD-2008-MI-00992	*L. monocytogenes*	1/2a	CFIA Dartmouth Lab	Sliced roast beef	L. mono (2)
3. 2157	*L. monocytogenes*	4b	CFIA Dartmouth Lab	Pepper salami	L. mono (3)
4. DAR-FD-2008-MI-01206	*L. monocytogenes*	3a	CFIA Dartmouth Lab	Cold smoked Salmon	L. mono (4)
5. DAR-FD-2009-MI-00615	*L. monocytogenes*	1/2a	CFIA Dartmouth Lab	Lima Bean	L. mono (5)
6. 2117	*L. monocytogenes*	4b	CFIA Dartmouth Lab	Soft Cheese	L. mono (6)

7. HPB-5764	*L. monocytogenes*	1/2c	CFIA Dartmouth Lab	Environment	L. mono (7)
8. HPB-3578	*L. monocytogenes*	3a	CFIA Dartmouth Lab	Environment	L. mono (8)
9. HPB-2442	*L. monocytogenes*	3b	CFIA Dartmouth Lab	Environment	L. mono (9)
10. FSL-J1-049	*L. monocytogenes*	3c	CFIA Dartmouth Lab	Human sporadic	L. mono (10)
11. FSL-J1-031	*L. monocytogenes*	4a	CFIA Dartmouth Lab	Human sporadic	L. mono (11)
12. FSL-W1-110	*L. monocytogenes*	4c	CFIA Dartmouth Lab	Unknown	L. mono (12)
13. Lm-F-117	*L. monocytogenes*	ST9	Dr. Gustavo Mallo at the Toronto Laboratories of Ontario Agency for Health and Prevention	Food products	N/A
14. Lm-F-92	*L. monocytogenes*	ST5	Dr. Gustavo Mallo at the Toronto Laboratories of Ontario Agency for Health and Prevention	Frozen dairy product	N/A
15. 1996-72	*L. innocua*	N/A	CFIA Dartmouth Lab	Meat Swab	L. inno (1)
16. 1996-74	*L. innocua*	N/A	CFIA Dartmouth Lab	Meat Swab	L. inno (2)
17. 1996-115	*L. innocua*	N/A	CFIA Dartmouth Lab	Swab	L. inno (3)

18. 1996-143	*L. innocua*	N/A	CFIA Dartmouth Lab	Wooden pole	L. inno (4)
19. 1996-148	*L. innocua*	N/A	CFIA Dartmouth Lab	Floor and Drain	L. inno (5)
20. 1996-150	*L. innocua*	N/A	CFIA Dartmouth Lab	Pork Sausage	L. inno (6)
21. 1996-268-1	*L. innocua*	N/A	CFIA Dartmouth Lab	Gouda cheese	L. inno (7)
22. DAR-FD-2000-MI-00156	*L. innocua*	N/A	CFIA Dartmouth Lab	Strawberry	L. inno (8)
23. NO ID (1)	*L. innocua*	N/A	CFIA Dartmouth Lab	Frozen peas	L. inno (9)
24. NO ID (2)	*L. innocua*	N/A	CFIA Dartmouth Lab	Corn	L. inno (10)
25. 2007-663	*L. innocua*	N/A	CFIA Dartmouth Lab	Unknown	L. inno (11)
26. NO ID (3)	*L. innocua*	N/A	CFIA Dartmouth Lab	Raw milk	L. inno (12)

3.2 Preparation of Cultures

Prior to each experiment, one colony of each strain was inoculated into 3 ml of brain-heart infusion (BHI) broth (Difco) and incubated at 30°C for 24 hours (Isotemp™ incubator, Fisher Scientific, Ottawa, Ontario, Canada) without shaking. These growth conditions were used to reduce the overgrowth of cultures. Optical density (OD) was read at 600 nm in a spectrophotometer (BioPhotometer®, Eppendorf, Mississauga, Ontario, Canada) for all cultures after the 24 hour growth period. Then, according to the OD reading, the density of the bacterial culture was adjusted to obtain a suspension with an OD of 1 unit, which was assumed to be equivalent to 10^9 CFU/ml. Cultures were then diluted further in either BHI broth, UVM1 broth or

peptone water to obtain the desired level of CFU/ml, as specified in the corresponding sections below.

3.3 Compounds

Phytochemicals and synthetic chemicals used in this study are listed in Table 2-4. These compounds were prepared for experiments by dissolving in dimethyl sulfoxide (DMSO) (Fisher Scientific, Ottawa, Ontario, Canada) to create a stock solution of 4 mg/ml. To achieve the desired working concentrations, stock solutions were diluted in two different media; UVM1 for the efflux pump inhibitors, and BHI for the general antimicrobials, such that a maximum concentration of 10% DMSO was obtained. Essential oils were stored as full strength (100%) solutions and then diluted to desired concentrations with a 0.15% agar solution (Difco, Agar, Granulated) as described by Walmiki and Rai (2017).

Table 2: List of individual phytochemicals with their source and known antimicrobial properties

Phytochemical	Company and Catalog #	Antimicrobial property	Reference
Ferruginol	Sigma-Aldrich 47714	Efflux pump inhibitor	Smith et al, 2007
Piperine	Sigma-Aldrich P49007	Efflux pump inhibitor	Mirza et al, 2011
Silibinin	Sigma-Aldrich S0417	Efflux pump inhibitor	Lee et al, 2012
Saponins from Quillaja Bark	Sigma- Aldrich S7900	General antimicrobial	Tagousop et al, 2018
Carvacrol	Sigma-Aldrich W224502	Increase membrane permeability	Xu et al, 2008
Quercetin	Sigma-Aldrich 1592409	General antimicrobial	Wang et al, 2018
Cinnamyl Alcohol	Sigma-Aldrich W228613	CdeA inhibition	Albano et al, 2018
Trans-Ferulic Acid	Sigma-Aldrich 128708	General antimicrobial	Shi et al, 2016
1,4, Napthoquinone	Sigma-Aldrich 152757	General antimicrobial	Janeczko et al, 2016
Gallic Acid	Sigma-Aldrich G7384	Increase membrane permeability	Sorrentino et al, 2017

Theaflavin (≥90% Purity)	Sigma-Aldrich 55016	General antimicrobial	Friedman et al, 2006
Thymol	Sigma-Aldrich T0501	Increase membrane permeability	Xu et al, 2008
Eugenol	Sigma-Aldrich E51791	General antimicrobial	Mak et al, 2019
Berberine	Sigma-Aldrich B3251	General antimicrobial	Xie et al, 2012
Theaflavin (97% Purity)	ChromaDex ASB-00020252-010	General antimicrobial	Friedman et al, 2006

Table 3: List of synthetic chemicals used with their source and known antimicrobial property

Synthetic Chemicals	**Company and Catalog #**	**Antimicrobial property**	**Reference**
Carbonyl cyanide 3-chlorophenylhydrazene (CCCP)	Sigma-Aldrich C2759	Efflux pump inhibitor	Baron and Rolain,2017
1-(1-Naphthymethyl) piperazine (NMP)	Sigma-Aldrich 651699	Efflux pump inhibitor	Sonnet et al, 2011
Chloropromazine hydrocholoride	Sigma-Aldrich C8138	Efflux pump inhibitor	Lechner et al, 2008
Omeprazole	Sigma-Aldrich O104	Efflux pump inhibitor	Vidaillac et al, 2006
Verapamil hydrochloride	Sigma-Aldrich V4629	Efflux pump inhibitor	Adams et al, 2014
Indole-3-carbinol	Sigma-Aldrich I7256	General antimicrobial	Sung and Lee, 2008

Table 4: List of essential oils used with their source and known antimicrobial property

Essential Oil	**Company and Catalog #**	**Antimicrobial property**	**Reference**
Fennel (sweet)	doTerra 41291813	Increase membrane permeability	Roby et al, 2012
Lavender	doTerra 30111813	Increase membrane permeability	Marín et al, 2016
Oregano	doTerra 30181813	Increase membrane permeability	Rodriguez-Garcia et al, 2016
Tea Tree	doTerra 30152113	Increase membrane permeability	Cox et al, 2000

Cinnamon Bark	doTerra 30031813	Increase membrane permeability	Wang et al, 2018b
Cassia	doTerra 30021813	Increase membrane permeability	Cunha et al, 2013
Peppermint	doTerra 30192113	Increase membrane permeability	Marwa et al, 2017
Thyme	doTerra 30221813	Increase membrane permeability	Sarengaowa et al, 2019
Coriander	doTerra 30781813	Increase membrane permeability	Silva et al, 2011
Anise	Sigma-Aldrich 10521	Increase membrane permeability	Abdel-Reheem and Oraby, 2015

3.4 Determination of Minimum Inhibitory Concentrations (MIC)

Utilizing *L. monocytogenes* strain Lm-F-117, the MIC for all phytochemicals was determined. The strain was used as it was previously determined to be a robust biofilm producer using the crystal violet microtiter plate assay (Stepanovic et al, 1999), potentially leading to higher resistance to the phytochemicals under examination. In a 96-well polystyrene plate (COSTAR), 200 µl of each compound at 400 µg/ml was added to the first well of a row, and depending on the tested chemical (i.e efflux pump inhibitor or general antimicrobial), 100 µl of either BHI broth or UVM1 broth was added to the remaining wells in the row. BHI broth was used for compounds with general antimicrobial properties and UVM1 broth was used for compounds known to have efflux pump inhibitor activity. Two-fold serial dilutions were performed by sequentially transferring 100 µ1 from each well to the next, with the last removal of 100 µ1 being discarded. For the positive control, 200 µl solution of 10% DMSO was added to the first well in the row and the remaining wells containing 100 µl of BHI broth or UVM1 broth, followed by the two-fold serial dilution as described above. Wells containing 200 µl of non inoculated BHI broth or UVM1 broth were considered negative controls. After serial dilution was performed, 100 µl of 10^7 CFU/ml culture was added to each well for a total volume of 200 µl.

This level of inoculation was utilized to ensure the growth of *L. monocytgogenes* occurred within 24 hours. Cultures were diluted in either BHI broth or UVM1 broth depending on the compound being used as mentioned above. The 96-well plate was then inserted into a microplate reader (CLARIOstar, BMG Labtech, Guelph, Ontario, Canada) at 35°C for 23 hours, where OD readings for each well were automatically taken every hour at 600 nm for a total of twenty-four data points. This temperature was used as it is a recommended temperature for the incubation of *L. monocytogenes* in UVM broth.

3.5 Initial Screening of Phytochemicals against *L. monocytogenes* and *L. innocua*

Utilizing *L. monocytogenes* strain FSL-J1-031 and *L. innocua* strain 2007-663, a high and low dose was selected per compound based on the results of the MIC experiment previously described above. The *L. monocytogenes* strain was chosen due to the fact that it was isolated from a human case, thus having relevance to human health. The *L. innocua* strain was chosen because it was the most recent strain isolated in the collection. In a 96-well polystyrene plate, 100 µl of each plant secondary metabolite was added to three wells at 2X the selected high concentration and three wells at 2X the selected low concentration for each *L. monocytogenes* and *L. innocua.* The high and low concentrations were chosen for each individual phytochemical used in these experiments are presented in Table 3 of the Results section below. The 96-well plate was then inoculated with 100 µl of either *L. monocytogenes* or *L. innocua* culture standardized to 10^7 CFU/ml to achieve a final volume of 200 µl in each well. Positive controls were designated as the wells containing 100 µl of the media plus 100 µl of the 10^7 CFU/ml culture (no phytochemicals added). Negative controls were designated as wells containing 200 µl of media (not inoculated with bacteria). The plate was incubated in the microplate reader at 35°C for 23 hours with OD readings of each well taken every hour at 600 nm. For all the essential oils tested, eugenol, and

berberine, the 96-well was set up in the same manner as described above; however, due to time constraint, the plates were incubated at 35°C in an incubator (Isotemp™ incubator, Thermo-Fisher) with periodic OD 600 nm readings taken at 0, 4, and 24 hours in the microplate reader and incubated at 35°C in the incubator.

Experiments were also set up to examine the synergistic effect of efflux pump inhibitors combined with other selected phytochemicals. As synergistic effects were expected, combinations of phytochemicals, 50 µl of each compound in the desired combination pairing was added to three wells for each *L. monocytogenes* and *L. innocua* at 4X the lowest dosage determined from the MIC results. Since efflux pump inhibitors were used, in all combinations, all compounds and cultures were diluted in UVM1 broth. Both *L. monocytogenes* and *L. innocua* cultures were diluted to 10^7 CFU/ml as before and 100 µl was added to the wells containing the combinations of phytochemicals for a final volume of 200 µl in each well. Positive and negative controls were designated as mentioned above. The 96-well plate was incubated at 35°C for 23 hours in the microplate reader as previously mentioned.

3.6 Screening of Phytochemicals against Multiple Strains of *Listeria*

Phytochemicals and/or their combinations that showed a differential effect between *L. monocytogenes* and *L. innocua* in the initial screening experiment were selected for screening against a larger contingent of strains consisting of twelve strains each of *L. monocytogenes* and *L. innocua*. In 96-well plates, three wells per strain contained 100 µl of phytochemicals or the combination as stated above. The wells then received 100 µl of 10^7 CFU/ml inoculum of the designated strain. An additional three wells per strain were considered positive controls and two wells per strain were assigned as negative controls and were set up as in the initial screening. To determine if the antimicrobial actions of the phytochemicals were bacteriostatic or bactericidal,

100 µl from each test well were spotted onto TSA agar and incubated at 30°C for 24 hours. The agar plates were removed and the spots were evaluated; if there was the presence of growth the effect was considered to be bacteriostatic and bactericidal if no growth occurred.

3.7 Determining Growth Kinetics of Milk Microbiota Biofilm

To determine the growth kinetics of the multispecies biofilm formation by microorganisms normally present in pasteurized milk was the crystal violet (CV) method of Stepanovic et al. (2000). was employed using polystyrene 96-well plates to quantify biofilm formation. The plate wells were filled with 200 µl of 2% Milk Fat (M.F) pasteurized milk and were kept at 4°C. The controls were: boiled milk that would serve as a negative control (little or no microbial biofilm formation expected), milk containing Cosmic serum™ (a complex mix of nutrients in high levels) which served as the positive control due to its promotion of higher microbial density growth was expected, and empty wells which served as blanks since no biofilm or layer of milk proteins would be present. Every two hours, duplicate wells were emptied, washed 3 times with 200 µl phosphate-buffered saline (PBS), and allowed to air dry. Once all the wells were empty, the biofilms were fixed by adding 200 µl of methanol (Caledon, Georgetown, Ontario, Canada) to each well for 15 minutes then allowed to air dry. The wells were then filled with 200 µl of 1% CV solution (Fisher Scientific, Ottawa, Ontario, Canada, C581-100) and left at room temperature for 5 minutes. The non-bound CV was removed with three washes with excess water. The bound CV was then re-solubilized in 200 µl of 33% acetic acid (Fisher Scientific, Ottawa, Ontario, Canada Fisher, SA36-1) per well and the absorbance measured at a wavelength of 570 nm, in the microplate reader. The amount of crystal violet was determined by a standard curve which is proportional to the amount of biomass present. (Appendix Figure 21 and 22).

3.8 The ability of Phytochemicals to Remove Preformed Biofilms In vitro

To determine whether the tested phytochemicals could remove preformed biofilms, 96-well microtiter plates were filled with 200 µl of 2% M.F pasteurized milk containing 100 CFU/ml of *L. monocytogenes.* The strain used was Lm-F-92 and was grown as described above in section 3.2. This *L. monocytogenes* strain was chosen as it was isolated from a frozen dairy product and therefore most likely to be characteristically similar to other strains found in milk. The culture was sufficiently diluted in peptone water to achieve a concentration of 100 CFU/ml when inoculated into the milk. The cell numbers for *L. monocytogenes* in the milk culture was confirmed by spotting 3X 100 µl onto Oxford selective agar and an additional 3X 100 µl onto Rapid L'mono selective agar and incubating at 35°C for 48 hours to allow the selective growth of *L. monocytogenes* colonies. The 96-well plate was then kept at 4°C for 74 hours based on the biofilm growth kinetics results. The milk was then removed, and the wells were washed 3X 200 µl PBS. The phytochemical solutions were then placed into the wells with three wells containing a high concentration and three wells containing a low concentration for each phytochemical solution and were left for 1-hour or 5-hour exposure. The solutions were then removed and washed again with 3X 200 µl PBS. The crystal violet procedure was then performed as described above in section 3.7 to determine the amount of biofilm removal compared to the controls (not exposed to phytochemicals) and the blank empty wells.

3.9 Dispensing equipment and pumps

Commercial milk dispensing units and pumps were utilized for the remaining experiments (Figure 1-3). The units contained reservoirs that held the milk or cleaning solutions. A length of silicone tubing connected the reservoir to the input portion of the pumps and an additional piece of silicone tubing was attached to the output of the pump and then to a stop valve where milk was

could be collected. Each dispensing unit had a refrigeration unit that allowed pumps, tubing, and reservoirs to remain at 4°C. For cleaning purposes (Figure 2), the stop valves were removed, and a long piece of Tygon tubing was attached to the pump output valve allowing liquids to empty back into the reservoir and be continuously circulated. The pumps sat within an automated drive that would automatically dispense 100 ml when engaged.

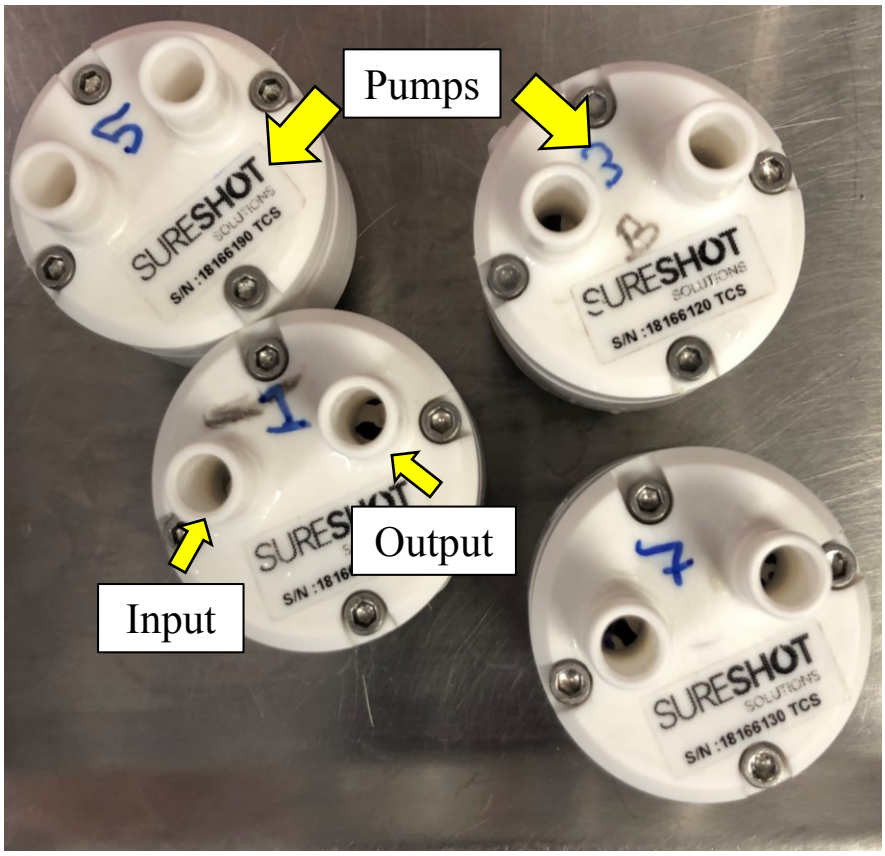


Figure 1: Front view of four pumps utilized throughout experiments with milk biofilm, showing the input and output ports. The diameter of these rotary pumps is approximately 5-6 cm.

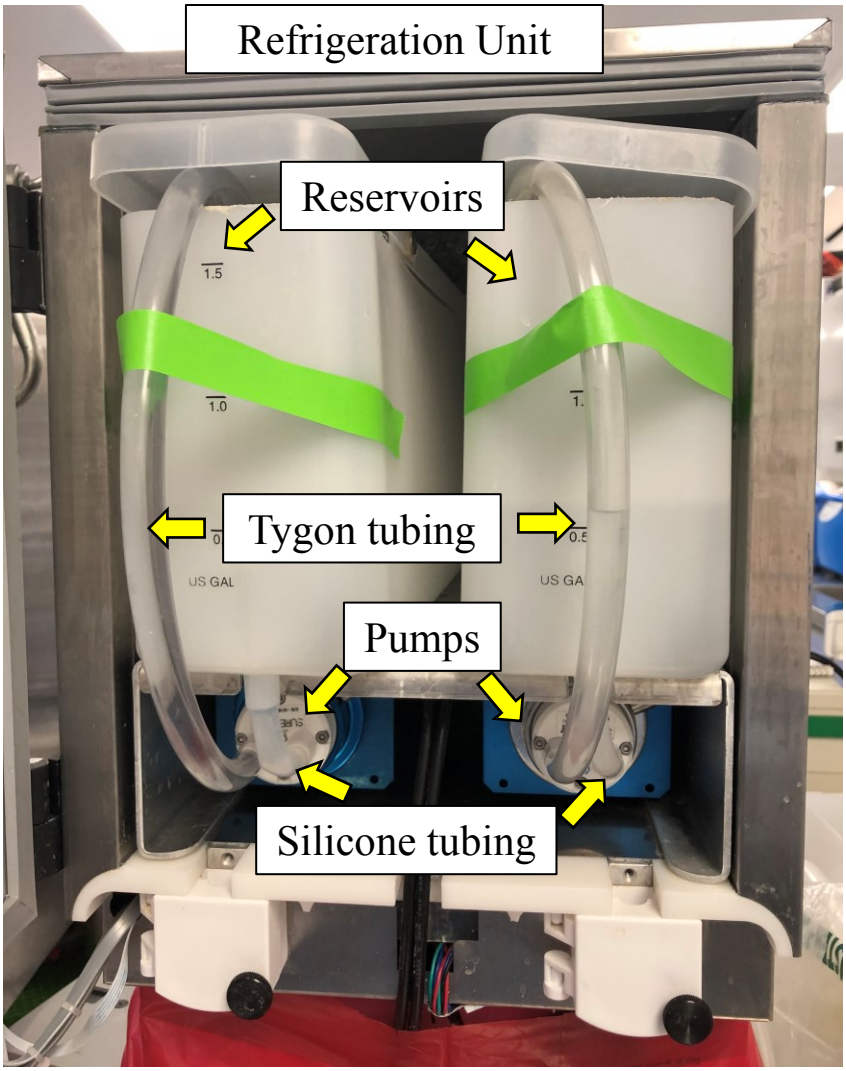


Figure 2: One dispensing units in their cleaning position containing two pumps indicating the position of the silicone tubing attached to the input and reservoirs, the Tygon tubing attached to the output with the other end in the reservoir, the pumps in the automatic drivers with all parts contained within a refrigeration unit.

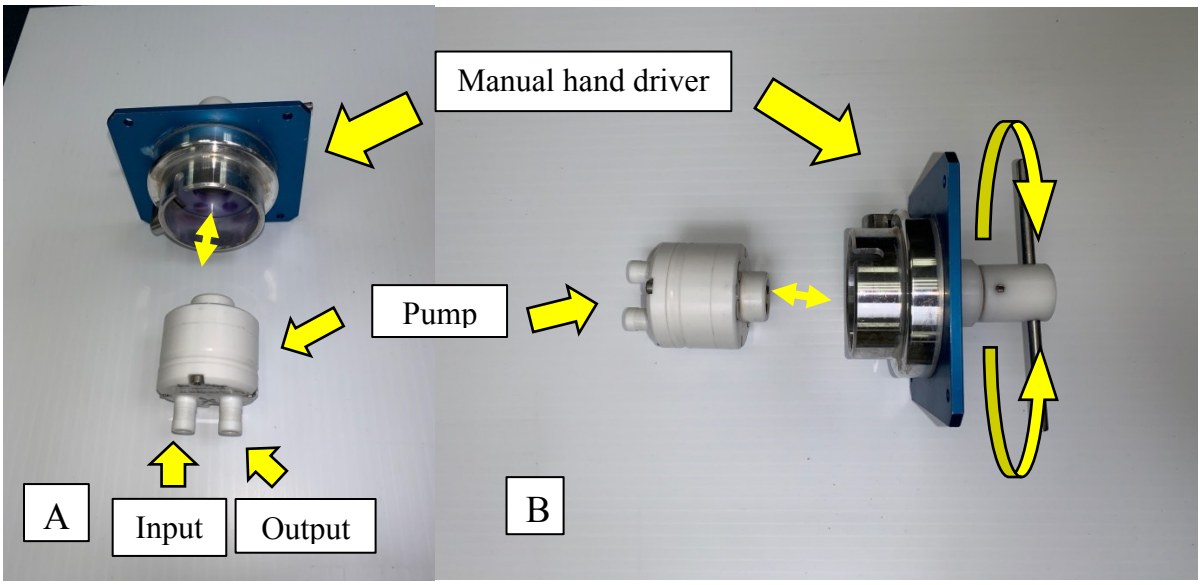


Figure 3: (A) Front view of manual hand driver showing how the pump fits into it. (B) Above view of manual hand driver and pump showing how the pump is turned when placed in the manual hand driver

3.10 Enumeration of *L. monocytogenes* in Dispensing Pumps

In order to determine if *L. monocytogenes* was able to harbor and replicate within the dispensing pumps, 2% M.F pasteurized milk was inoculated with *L. monocytogenes* strain Lm-F-92 at a final concentration of 1 CFU/ml. The duration of the experiment was 9 days with milk being replaced every 3 days. The pumps and milk within the reservoirs were kept at 4°C for the experiment. The *L. monocytogenes* inoculum was grown as previously described and diluted in peptone water to achieve 900 CFU/ml. The cell concentration of the inoculum was verified by spotting 100 µl onto TSA agar that was incubated at 30 °C for 24 hours. The dispensing machines contained a reservoir that was filled with 900 ml of 2% M.F pasteurized milk. To achieve a final listerial concentration in the milk of 1 CFU/ml, 1 ml of inoculum was transferred into the reservoir containing the milk. The milk was incorporated into the pumps by dispensing 100 ml of milk which was collected and discarded. Another 100 ml was dispensed, collected and

diluted 1:5 with peptone water, with 1 ml being spread evenly over 3 plates of Oxford selective agar and an additional 1 ml spread evenly over 3 plates of Rapid L'mono selective agar (according to method MFLP-74). The agar plates were incubated at 35°C for 48 hours and counted for *L. monocytogenes* colonies were enumerated to determine the number of CFU/ml. Every 12 hours, 100 ml of milk were dispensed and discarded until there was no milk left for another full 100-ml aliquot (72 hours). After the last 100 ml aliquot was collected, it was diluted and plated as described above to enumerate *L. monocytogenes*, and any remaining milk in the reservoir was removed and the reservoirs were detached from the pumps and tubing. The reservoirs were then submerged in 200 ppm bleach solution for 30 minutes. The pumps and tubing were left soiled throughout the duration of the experiment. The dispensing units were then reassembled and set up as described above for an additional 72 hours. The above procedure was completed again two more times for a total of 9 days.

3.11 Ability of Phytochemicals to Remove Preformed Biofilms in Dispensing Pumps

To establish a cleaning procedure for the commercial milk dispensing units, the pumps and tubing of the milk dispensing units were filled with 2% M.F pasteurized milk and left for 74 hours at 4°C for mature biofilm to form. The milk was replaced by the phytochemicals identified to remove biomass in the 96 well plates for an exposure time of 1 hour and at a high concentration. After the hour, the compounds were removed from the pump and attached silicone tubing and were washed 3X with 6 ml of PBS. To determine the degree of biofilm removal, 6 ml of 1% crystal violet was driven into the pumps with the manual drive, left for 15 minutes and then removed. The excess CV was washed away with water, and then 6 ml of 95% ethanol (Fisher Scientific, Ottawa, Ontario, Canada, 6000561), was added to the pumps using the manual drive, 15 minutes to sequester the stain. Ethanol was used in this step instead of acetic acid as

used above due to the acid having a negative effect on the impellers within the rotary pumps. The ethanol was then collected, and the absorbance was read in the spectrophotometer at 562 nm. A control pump was included that was not exposed to phytochemicals for comparison. The concentration of CV was then determined by a standard curve, allowing a correlation to the amount of biomass remaining.

3.12 Ability of Phytochemicals to Remove Preformed Biofilms in Dispensing Pumps with Mechanical Actions

To determine if phytochemicals with the addition of mechanical action could remove more preformed biofilms in the dispensing pumps, one colony of Lm-F-92 was inoculated into 3 ml of BHI broth and incubated at 30°C for 2 hours. The full incubation time was not utilized to verify *L. monocytogenes*' ability to grow in milk. Utilizing 25ml of 2% M.F pasteurized milk, 1ml of the inoculum was dispensed into the milk. Using the manual hand driver (Figure 2), each pump was filled with 6 ml of the inoculated milk, and a length of silicone tubing was attached to the input and output to ensure a closed environment. The pumps were kept at 4°C for 74 hours to allow for biofilm development. The milk was collected separately from each pump, and following the MFLP-74 method, it was diluted 1:5 with peptone water and 100 µl was spotted onto Oxford selective agar as well as Rapid L'mono selective agar, which were incubated at 35°C for 48 hours to determine if *L. monocytogenes* was present in the milk. Once the milk was removed, the pumps and silicon tubing were rinsed 3X with 6 ml of PBS. The dispensing units were set up for cleaning, with the pumps inserted in the automatic drivers, the silicone tubing attached to the input of the pump at one end and the reservoir at the other end and Tygon tubing attached to the output of the pump at one end and the other end within the reservoir. Utilizing thymol and carvacrol, the compounds were diluted to the desired concentration with peptone

water with the final volume being 200ml being added to each reservoir. The pumps were then run for 15 minutes, rested for 15 minutes and then ran for an additional 15 minutes with the compound circulating throughout the system when the pumps were running. When the cleaning cycle was complete the pumps were removed from the dispensing units and were washed 3X with 6 ml of PBS. To determine the degree of biofilm removal, the CV assay was conducted at described in section 3.11.

3.13 Statistical Analysis

The Student t-test was used to determine significant differences between *L. monocytogenes* and *L. innocua* during the initial screening of the phytochemicals as well as to determine any significant differences between the biomass of the 2% M.F pasteurized milk, the 2% M.F pasteurized milk with Cosmic Serum and the boiled 2% M.F pasteurized milk. Results were considered significant when P was a value <0.05. The ANOVA test was used to determine the significant difference between the twenty-four strains of *Listeria*, again results were considered significant when P was a value <0.05. The calculator design by R.Lowry at Vassar College, Poughkeepsie, NY, USA (http://vassarstats.net/) was used for both the Student t-test and the ANOVA test.

CHAPTER 4 RESULTS

4.1 All phytochemicals and synthetic efflux pump inhibitors tested had some anti-listerial activity

The minimum inhibitory concentration was determined for each phytochemical (Table 3) against *L. monocytogenes* strain Lm-F-117, in order to choose a high and low concentration for comparative growth trails between *L. monocytogenes* and *L. innocua*. MICs for phytochemicals were determined and found to be in a range from 0.075 µg/ml to 200 µg/ml. Phytochemicals with general antimicrobial properties were determined to have the following MICs: 200 µg/ml for theaflavin (97% Purity), eugenol and berberine; 100 µg/ml for carvacrol, quercetin, trans-ferulic acid, gallic acid, thymol, indole-3-carbinol, and saponins; 75 µg/ml for cinnamyl alcohol; 25 µg/ml for 1,4 naphthoquinone; and 12.5 µg/ml for theaflavin (≥90% Purity). For screening the effect of the phytochemicals against *L. monocytogenes* and *L. innocua*, high and low concentrations above(but not beyond 200 µg/ml) and below the MIC concentration of strains Lm-F-117 were selected as follows; theaflavin (97% Purity), eugenol and berberine, 200 µg/ml and 100 µg/ml; carvacrol, quercetin, trans-ferulic acid, gallic acid, thymol, indole-3-carbinol and saponins, 200 µg/ml and 50 µg/ml; cinnamyl alcohol, 100 µg/ml and 50 µg/ml; 1,4 naphthoquinone, 50 µg/ml and 12.5 µg/ml; and theaflavin (>90% Purity), 25 µg/ml and 6.25 µg/ml. Phytochemicals known to be efflux pumps inhibitors displayed had a range of MICs as follows: ferruginol, and silibinin, 100 µg/ml; piperine and NMP, 50 µg/ml; verapamil, chloropromazine hydrochloride, and omeprazole, 6.25 µg/ml; and CCCP, 0.78 µg/ml. The chosen high and low concentrations for these efflux pump inhibitors were 200 µg/ml and 50 µg/ml for ferruginol and silibinin; 100 µg/ml and 25 µg/ml for piperine and NMP; 12.5 µg/ml and 3.125 µg/ml for verapamil, chloropromazine hydrochloride and omeprazole; and 1.56 µg/ml and 0.39

μg/ml for CCCP. The MICs for the essential oil compounds were determined to be 0.2 μg/ml for lavender, peppermint, fennel, tea tree, and coriander; and 0.075 μg/ml for cinnamon bark. Therefore, the chosen high and low concentrations for the essential oils were 0.2 μg/ml and 0.1 μg/ml for lavender, peppermint, fennel, tea tree, and coriander; and 0.1 μg/ml and 0.05 μg/ml for cinnamon bark

Table 5: The minimum inhibitory concentration (MIC) of the compounds used against *L. monocytogenes* (Lm-F-117) and the chosen high and low concentration used for the initial screening against *L. monocytogenes* and *L. innocua*.

Compounds	**MIC (μg/ml)**	**High Concentration (μg/ml)**	**Low Concentration (μg /ml)**
Cinnamon Bark EO	0.075	0.1	0.05
Lavender EO	0.2	0.2	0.1
Peppermint EO	0.2	0.2	0.1
Fennel EO	0.2	0.2	0.1
Tea Tree EO	0.2	0.2	0.1
Coriander EO	0.2	0.2	0.1
CCCP	0.78	1.56	0.39
Chloropromazine hydrochloride	6.25	12.5	3.125
Omeprazole	6.25	12.5	3.125
Verapamil	6.25	12.5	3.125
Theaflavin (≥90% Purity)	12.5	25	6.25
1,4 Napthoquinone	25	50	12.5
Piperine	50	100	25
NMP	50	100	25
Cinnamyl Alcohol	75	100	50
Trans- Ferulic Acid	100	200	50
Thymol	100	200	50
Indole-3-carbinol	100	200	50
Saponins	100	200	50
Ferruginol	100	200	50
Silibinin	100	200	50
Gallic Acid	100	200	50
Carvacrol	100	200	50

Quercetin	100	200	50
Eugenol	200	200	100
Berberine	200	200	100
Theaflavin (97% Purity)	200	200	100

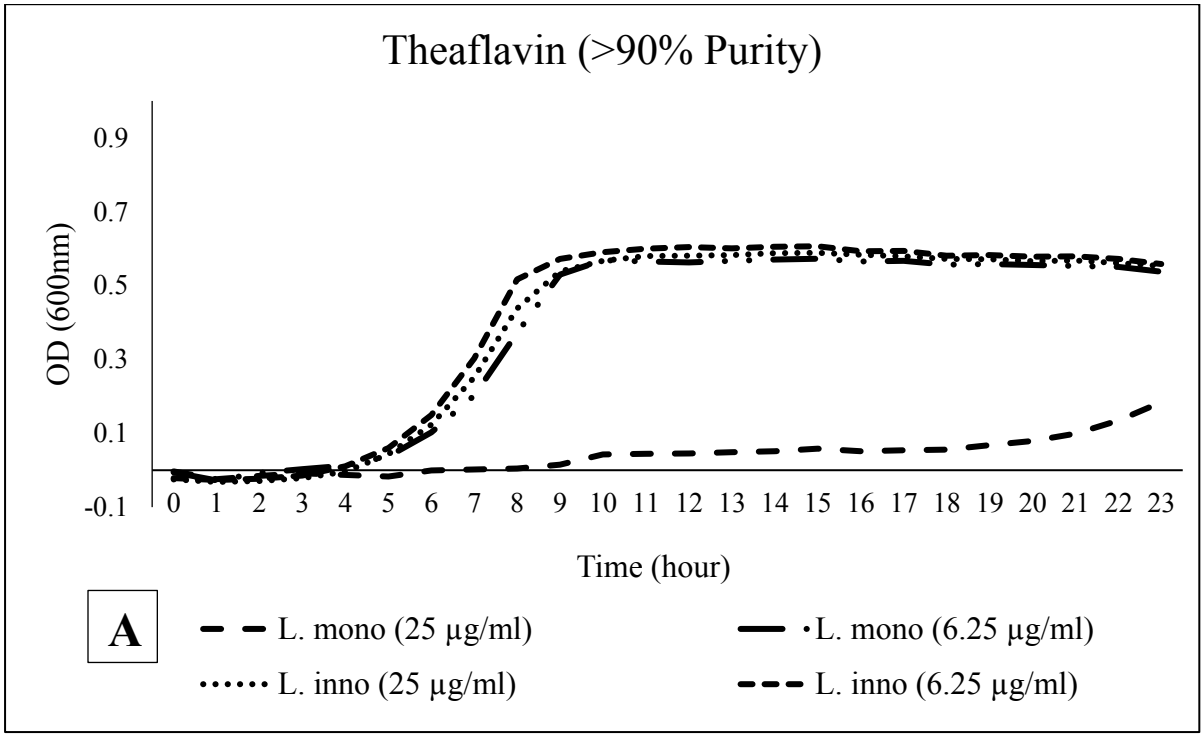
Abbreviations: EO: essential oil; NMP: 1-(1-Naphthymethyl)piperazine; CCCP: carbonyl cyanide 3-chlorophenylhydrazene

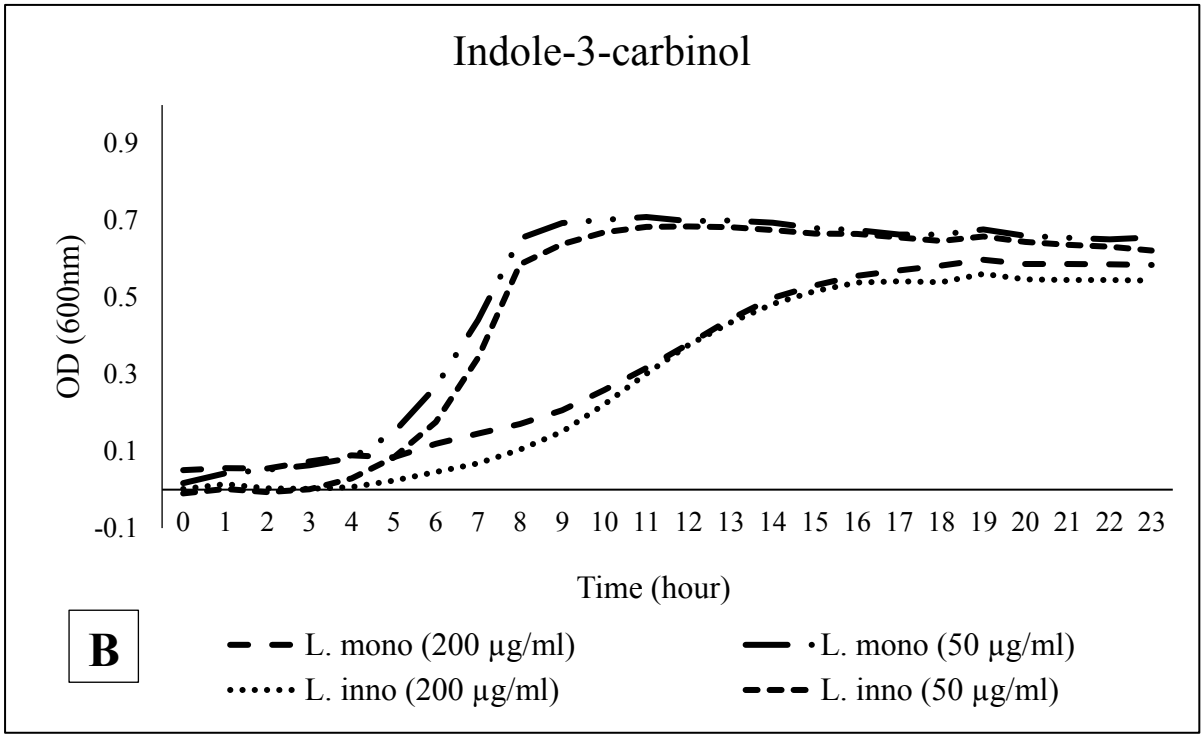
4.2 Fourteen Compounds and Five Combinations of Compounds Were Able to Show a Differential Effect Between *L. monocytogenes* and *L. innocua* After An Initial Screening

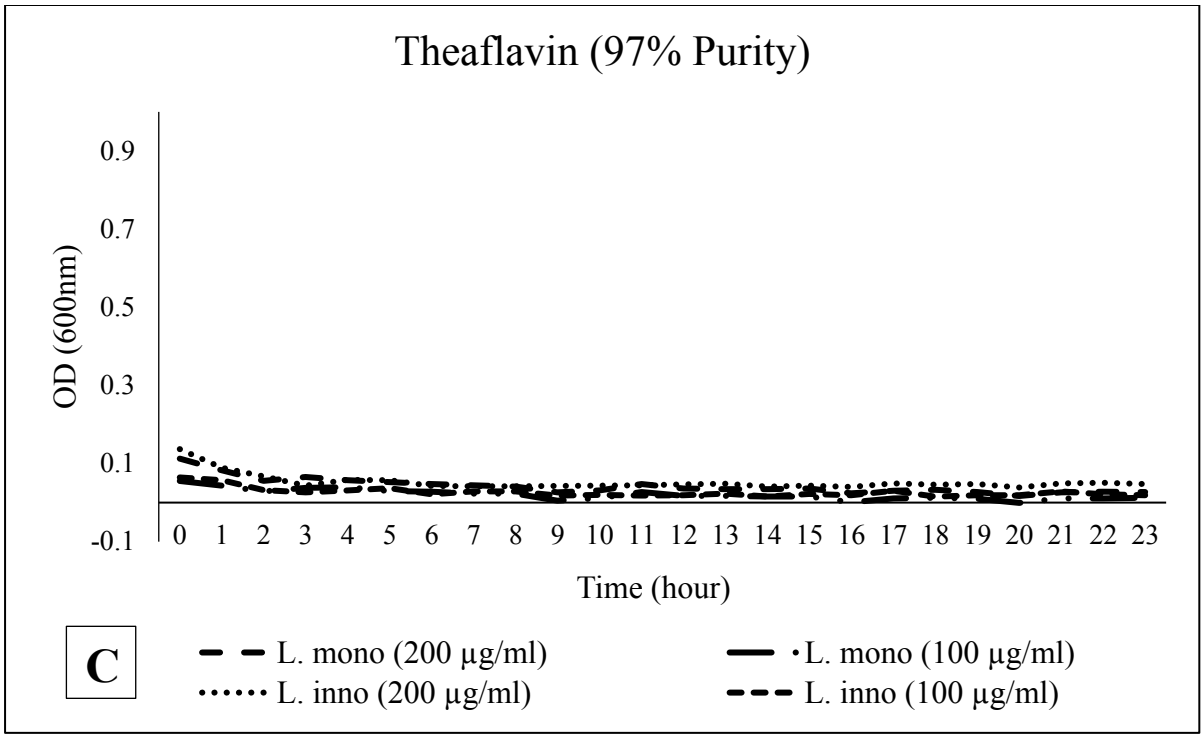
4.2.1 Only Five Individual Phytochemicals Showed a Differential Effect Between *L. monocytogenes* and *L. innocua*

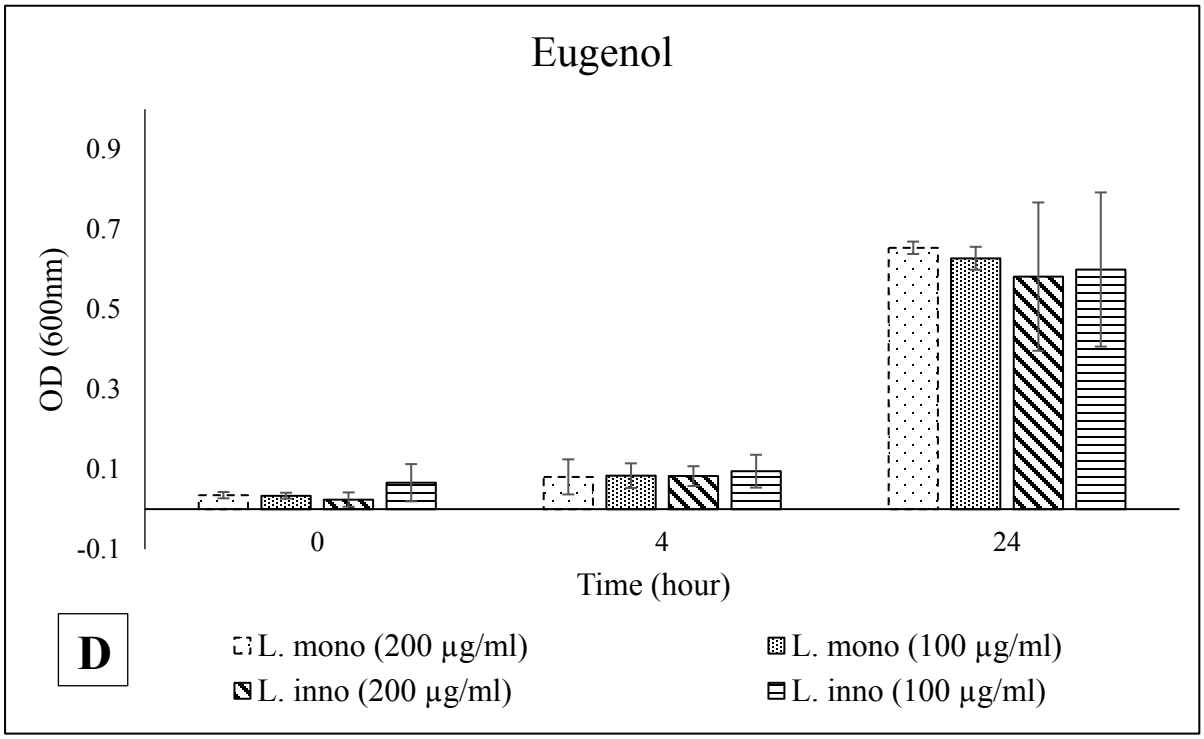
Utilizing *L. monocytogenes* strain FSL-J1-031 and *L. innocua* strain 2007-663, a high and low concentration of each phytochemical was selected to determine if there were a differential effect on growth between the two species of *Listeria*. A student t-test was used to determine if there was a statistical difference between the *Listeria* species growth curve, those with a significant difference ($P<0.05$) are shown in Figure 4-7 (Nonsignificant screenings in Appendix). The compounds with general antimicrobial properties that showed a significant differential effect were theaflavin (≥90% Purity) at 25 µg/ml (Figure 4A), indole-3-carbinol at 50 µg/ml (Figure 4B), theaflavin (97% Purity) at 200 µg/ml (Figure 4C), eugenol at 200 µg/ml and 100 µg/ml (Figure 4D) and berberine at 200 µg/ml (Figure 4E). Many compounds showed a significant ($P<0.05$) differential effect between the listeria species with more selecting *L. innocua* over *L. monocytogenes*. The compounds with general antimicrobial properties in BHI broth that showed a significant differential effect were theaflavin (≥90% Purity) at 25 µg/ml, indole-3-carbinol at 50 µg/ml, theaflavin (97% Purity) at 200 µg/ml, eugenol at 200 µg/ml and 100 µg/ml and berberine at 200 µg/ml

Initial Screening of Phytochemicals with General Antimicrobial Activity Against *L. monocytoges* and *L. innocua*






Theaflavin (97% Purity)
OD (600nm)
0.9
0.7
0.5
0.3
0.1
-0.1
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (hour)
C
L. mono (200 µg/ml)
L. mono (100 µg/ml)
L. inno (200 µg/ml)
L. inno (100 µg/ml)


Eugenol
OD (600nm)
0.9
0.7
0.5
0.3
0.1
-0.1
0
4
24
Time (hour)
D
L. mono (200 µg/ml)
L. mono (100 µg/ml)
L. inno (200 µg/ml)
L. inno (100 µg/ml)

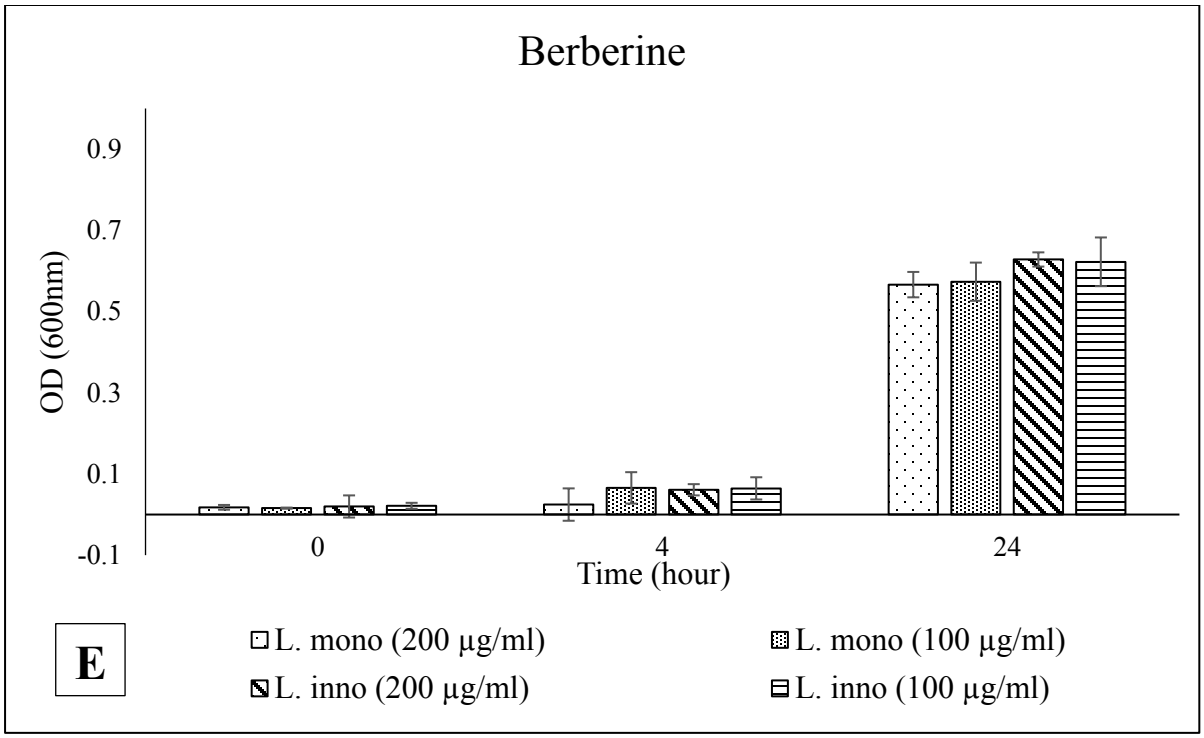
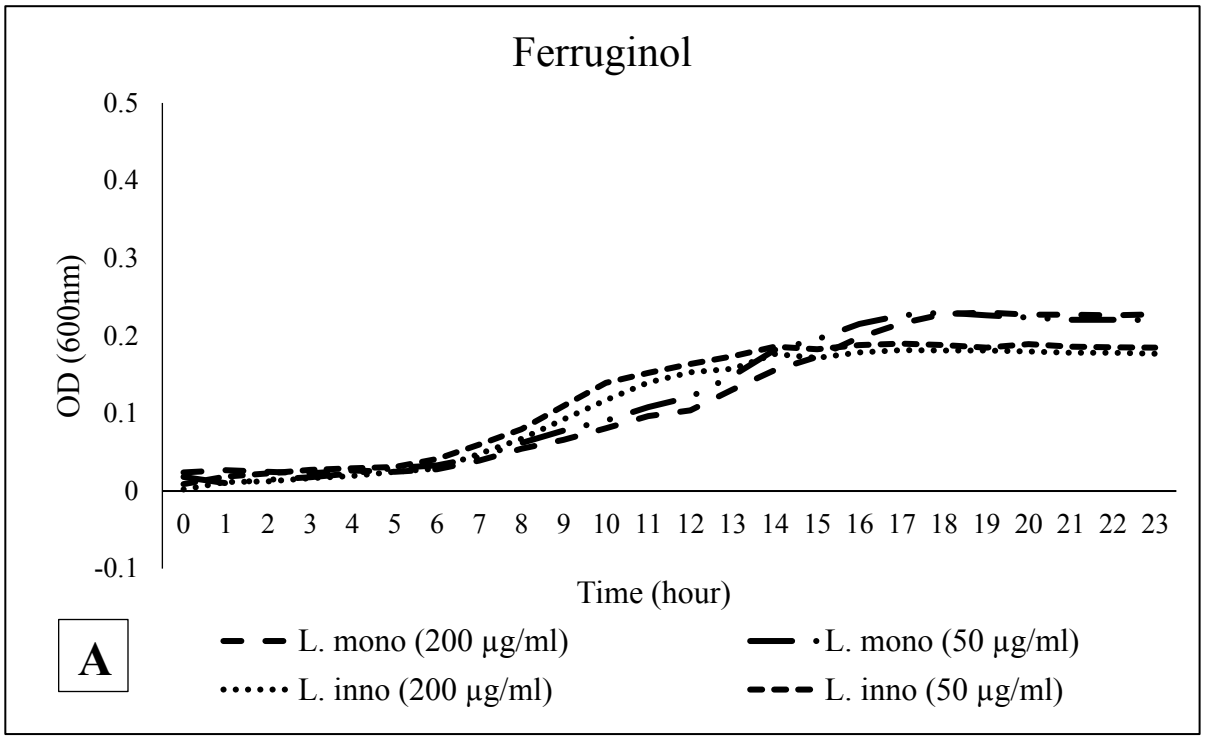


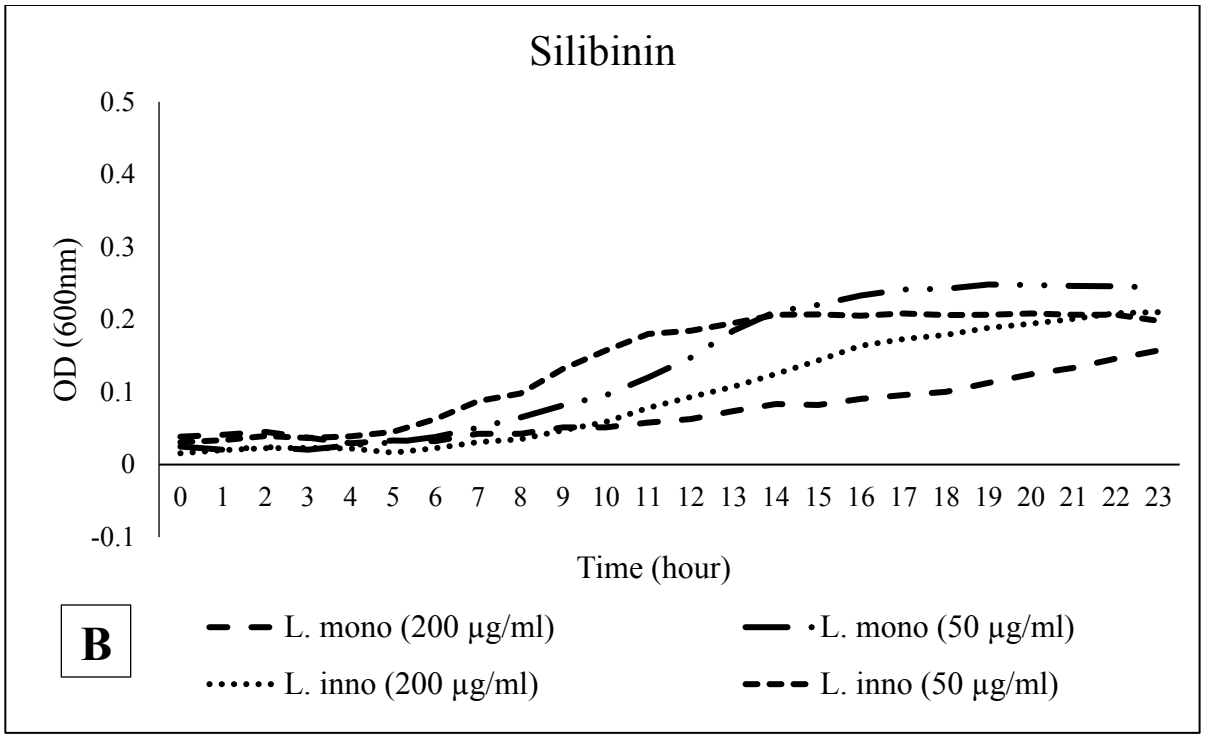
Figure 4: Growth curves (time vs OD_{600}) of the initial screening of (A) Theaflavin (≥90% Purity) at 25 µg/ml and 6.25 µg/ml, (B) Indole-3-carbinol at 200 µg/ml and 50 µg/ml, (C) Theaflavin (97% Purity) at 200 µg/ml and 100 µg/ml, and bar graphs of the initial screening of (D) Eugenol at 200 µg/ml and 100 µg/ml, and (E) Berberine at 200 µg/ml and 100 µg/ml against *L. monocytogenes* FSL-J1-031 and *L. innocua* 2007-663. Error bars indicate standard deviation. (n=9).

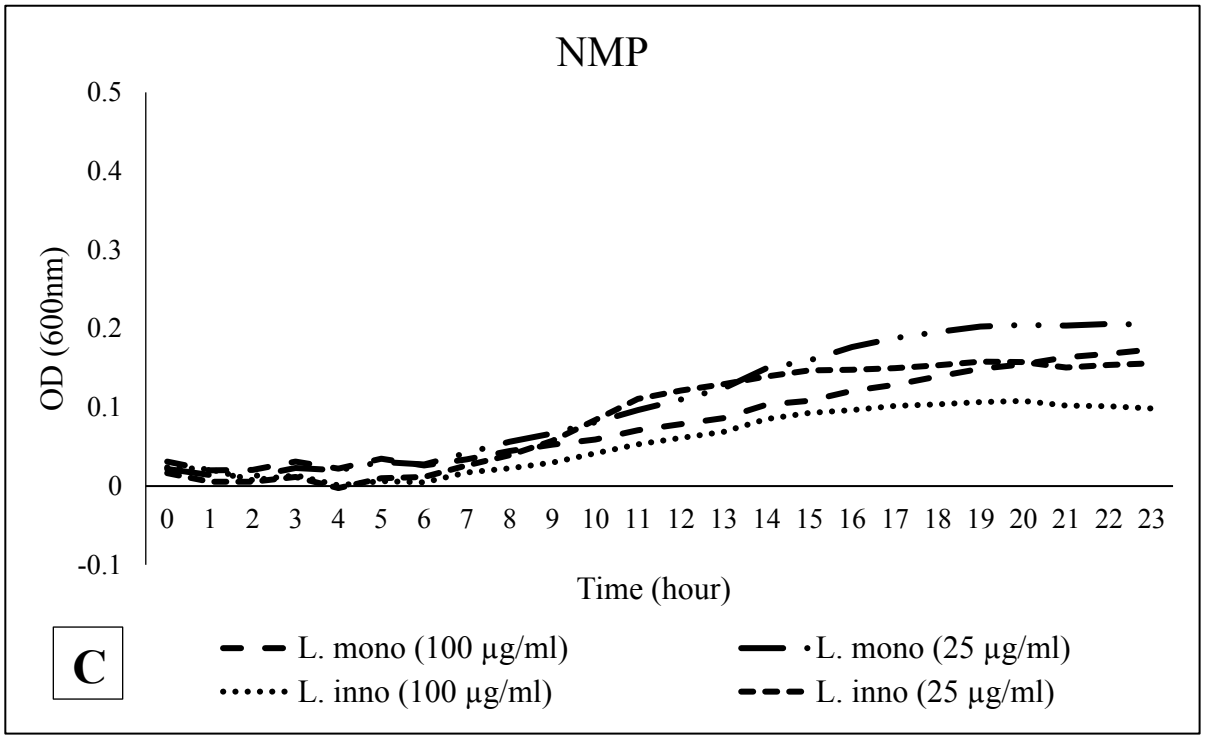
4.2.2 Four Efflux Pump Inhibitors Were Able To Have a Differential Effect Between *L. monocytogenes* and *L. innocua*

Four phytochemicals with known ability to inhibit efflux pumps that were tested in this study showed significantly differentiated growth the *L. monocytogenes* the *L. innocua* strains. These were ferruginol at 200 µg/ml (Figure 5A), silibinin at 50 µg/ml (Figure 5B), NMP at 100 µg/ml (Figure 5C), and omeprazole at 12.5 µg/ml (Figure 5D).

Initial Screening of Efflux Pump Inhibitor Phytochemicals Against *L. monocytogenes* and *L. innocua*




Silibinin
0.5
0.4
0.3
0.2
0.1
0
-0.1
OD (600nm)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (hour)
B
L. mono (200 µg/ml)
L. mono (50 µg/ml)
L. inno (200 µg/ml)
L. inno (50 µg/ml)


NMP
0.5
0.4
0.3
0.2
0.1
0
-0.1
OD (600nm)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (hour)
C
L. mono (100 µg/ml)
L. mono (25 µg/ml)
L. inno (100 µg/ml)
L. inno (25 µg/ml)

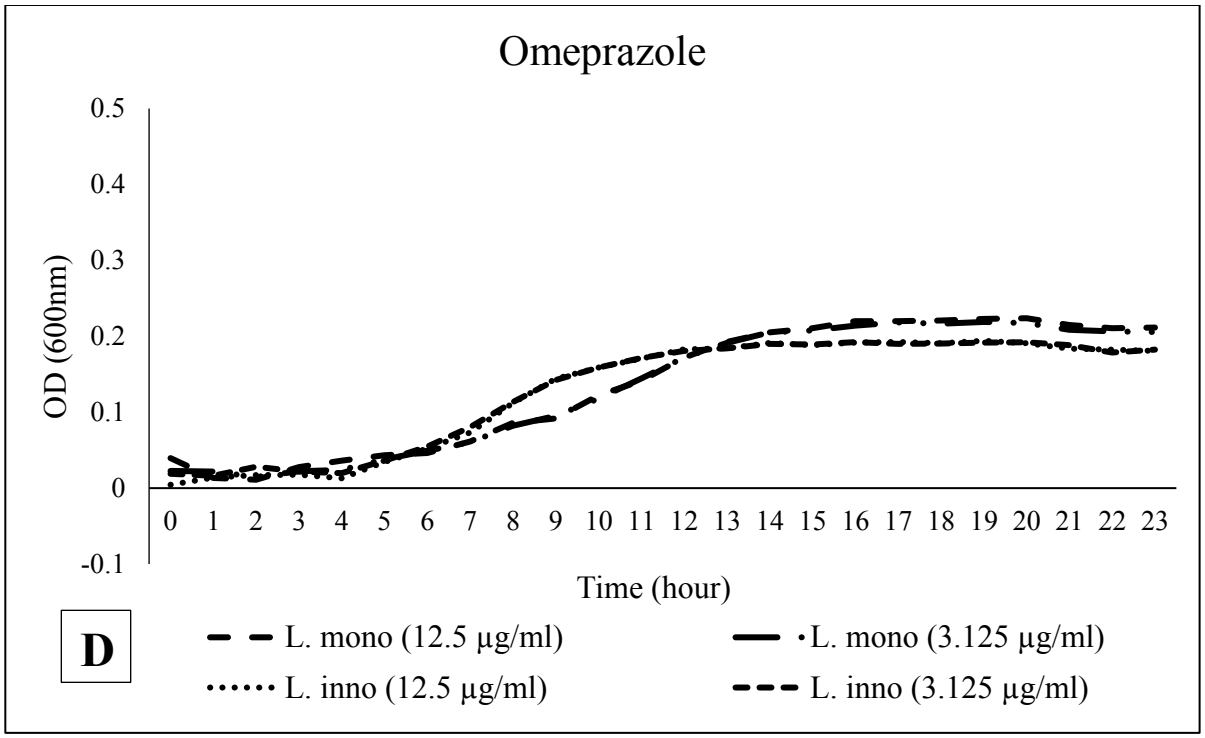
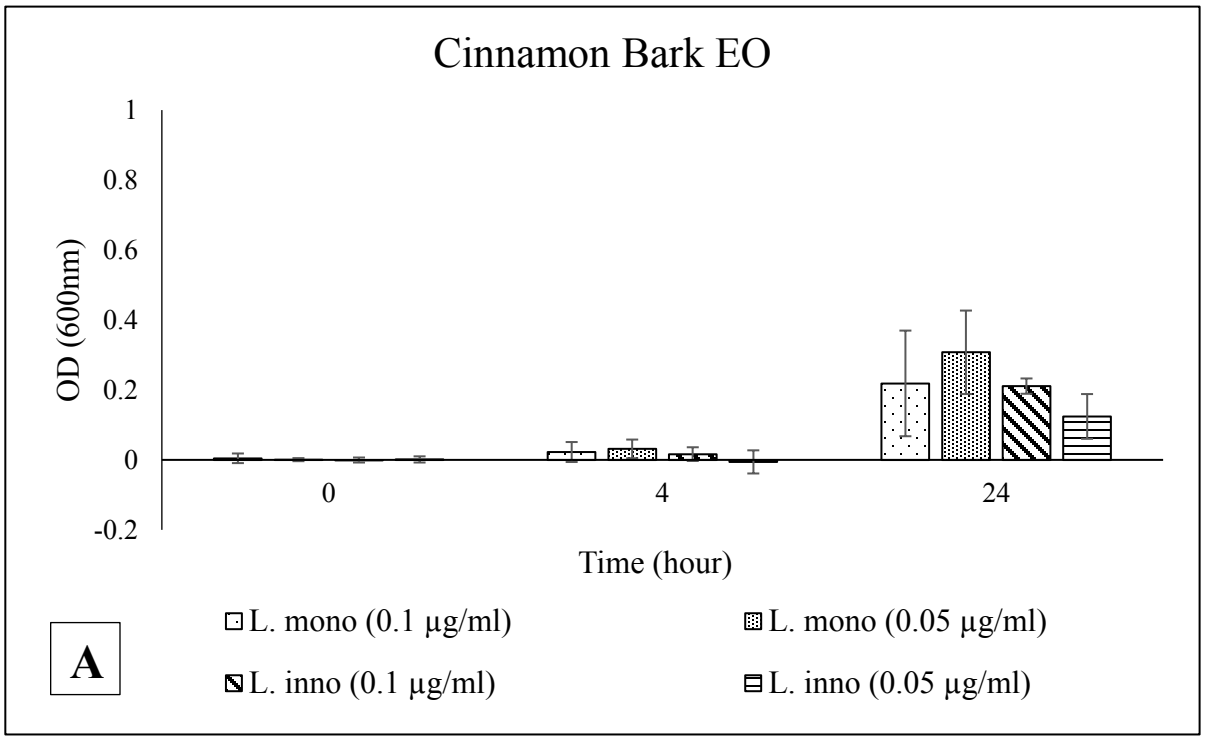


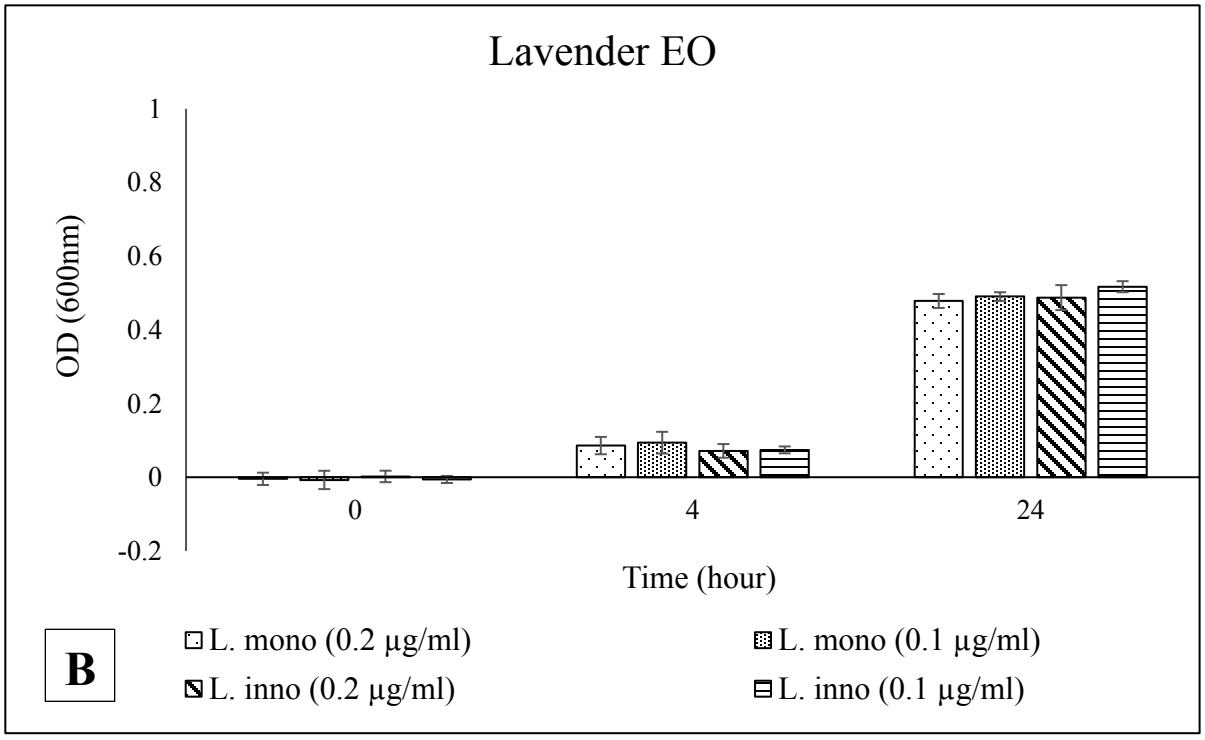
Figure 5: Growth curve (time vs OD_{600}) of the initial screening of (A) Ferruginol at 200 µg/ml and 50 µg/ml, (B) Silibinin at 200 µg/ml and 50 µg/ml, (C) NMP at 100 µg/ml and 25 µg/ml, and (D) Omeprazole at 12.5 µg/ml and 3.125 µg/ml against *L. monocytogenes* FSL-J1-031 and *L. innocua* 2007-663. (n=9).

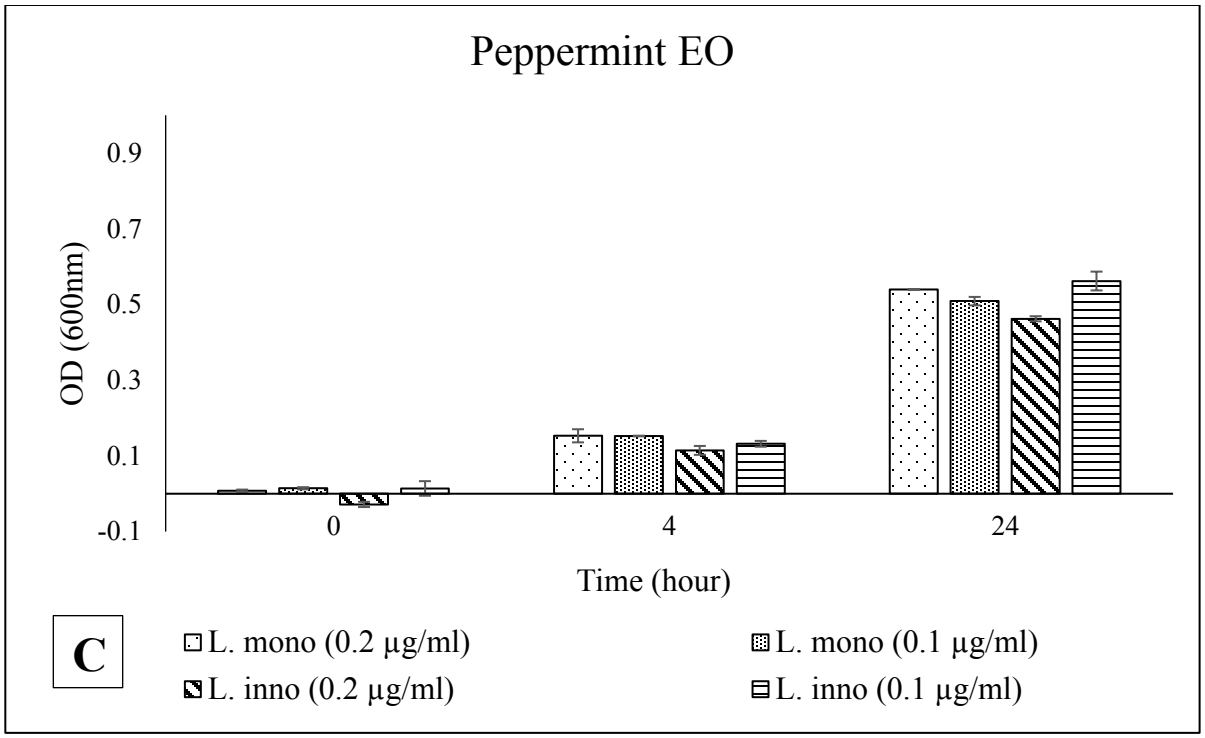
4.2.3 Five Essential Oils Were Able To Differentially Inhibit *L. monocytogenes* and *L. innocua*

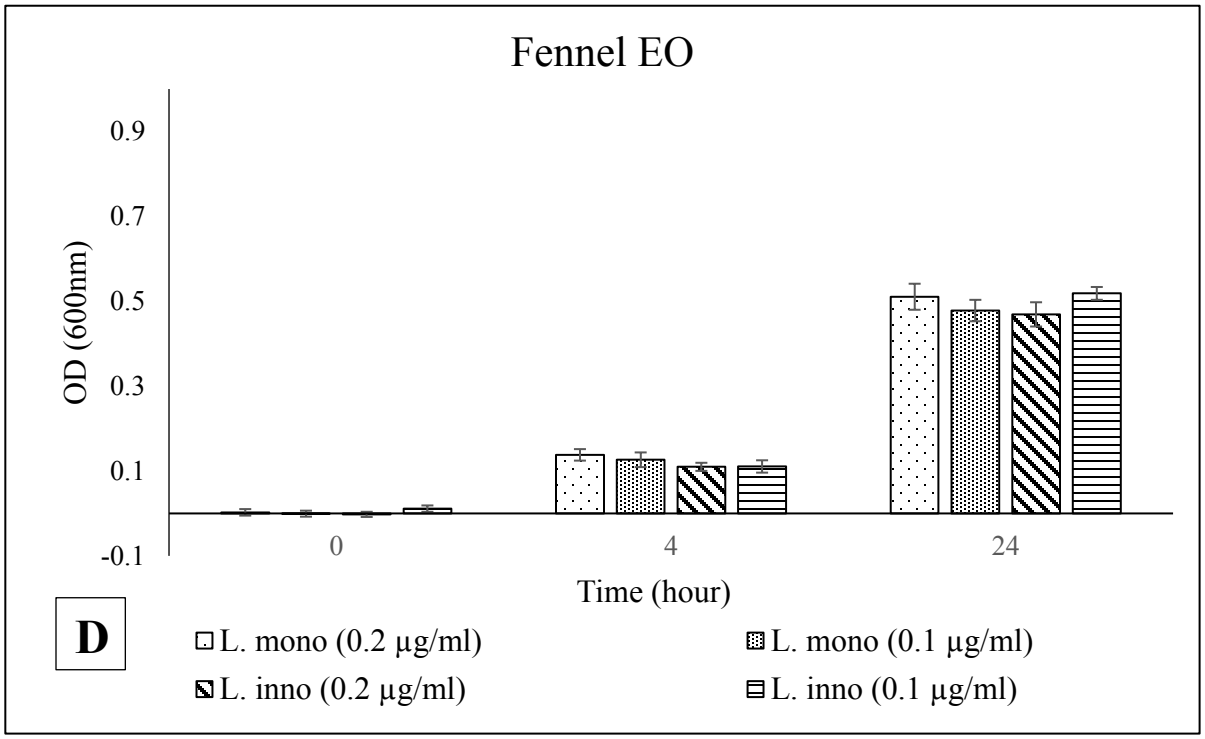
The same *L. monocytogenes* and *L. innocua* strains were also screened against essential oils with the following essentials oils having a significant differential effect between the two listeria species (Figure 6). Cinnamon bark EO at 0.05 µg/ml (Figure 6A), Lavender EO at 0.1 µg/ml (Figure 6B), peppermint EO at both 0.2 µg/ml and 0.1 µg/ml (Figure 6C), fennel EO at 0.1 µg/ml (Figure 6D), and coriander EO at 0.1 µg/ml (Figure 6E).

Initial Screening of Essential Oil Phytochemcials against *L. monocytogenes* and *L. innocua*






Peppermint EO
OD (600nm)
0.9
0.7
0.5
0.3
0.1
-0.1
0
4
24
Time (hour)
C
L. mono (0.2 µg/ml)
L. mono (0.1 µg/ml)
L. inno (0.2 µg/ml)
L. inno (0.1 µg/ml)


Fennel EO
OD (600nm)
0.9
0.7
0.5
0.3
0.1
-0.1
0
4
24
Time (hour)
D
L. mono (0.2 µg/ml)
L. mono (0.1 µg/ml)
L. inno (0.2 µg/ml)
L. inno (0.1 µg/ml)

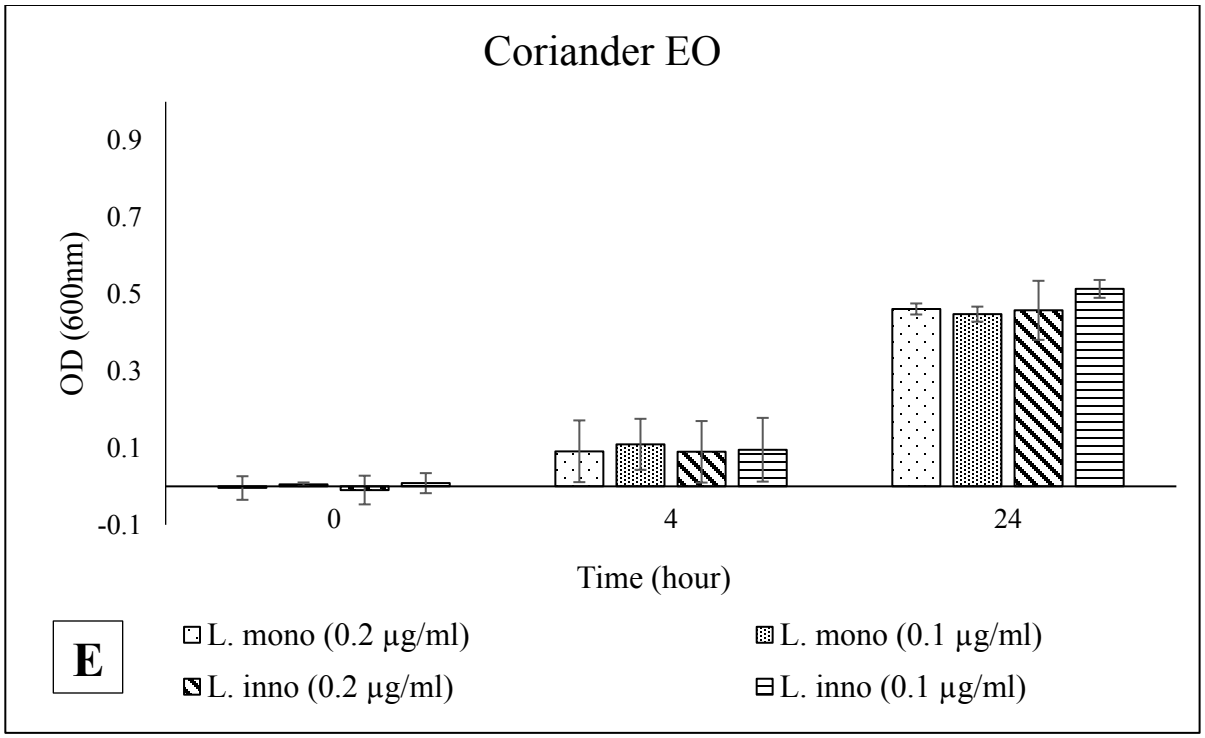
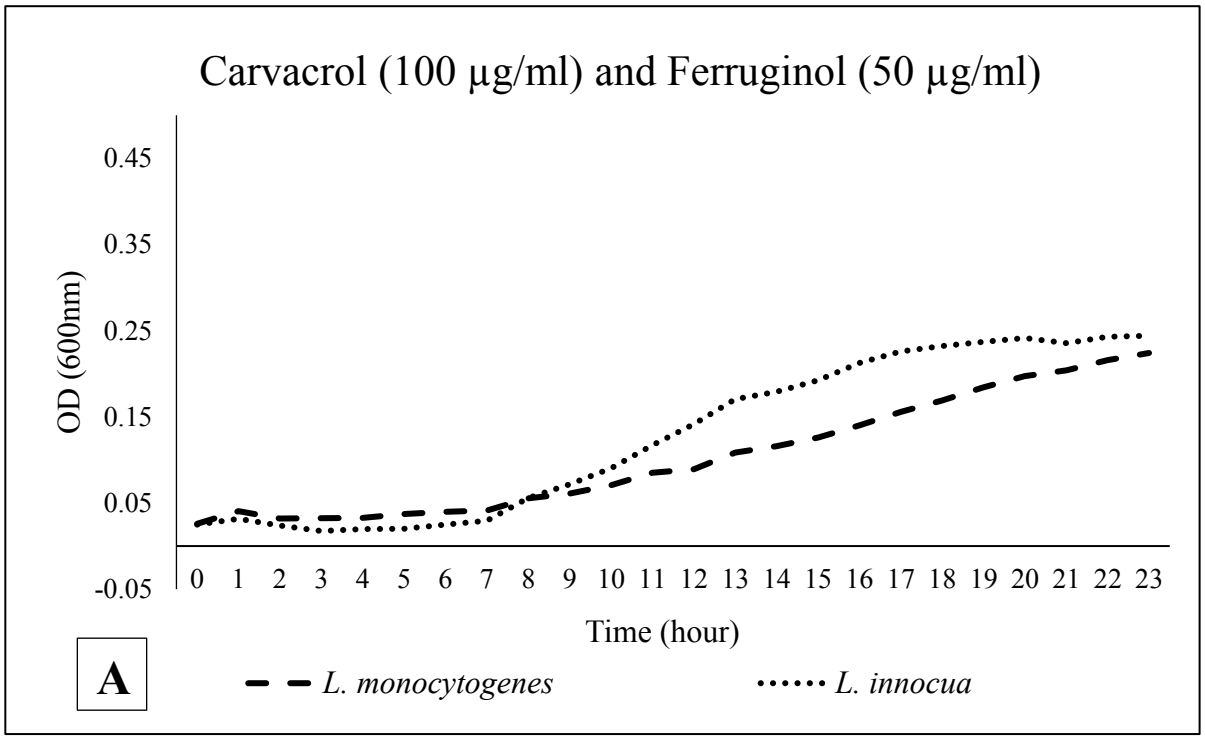


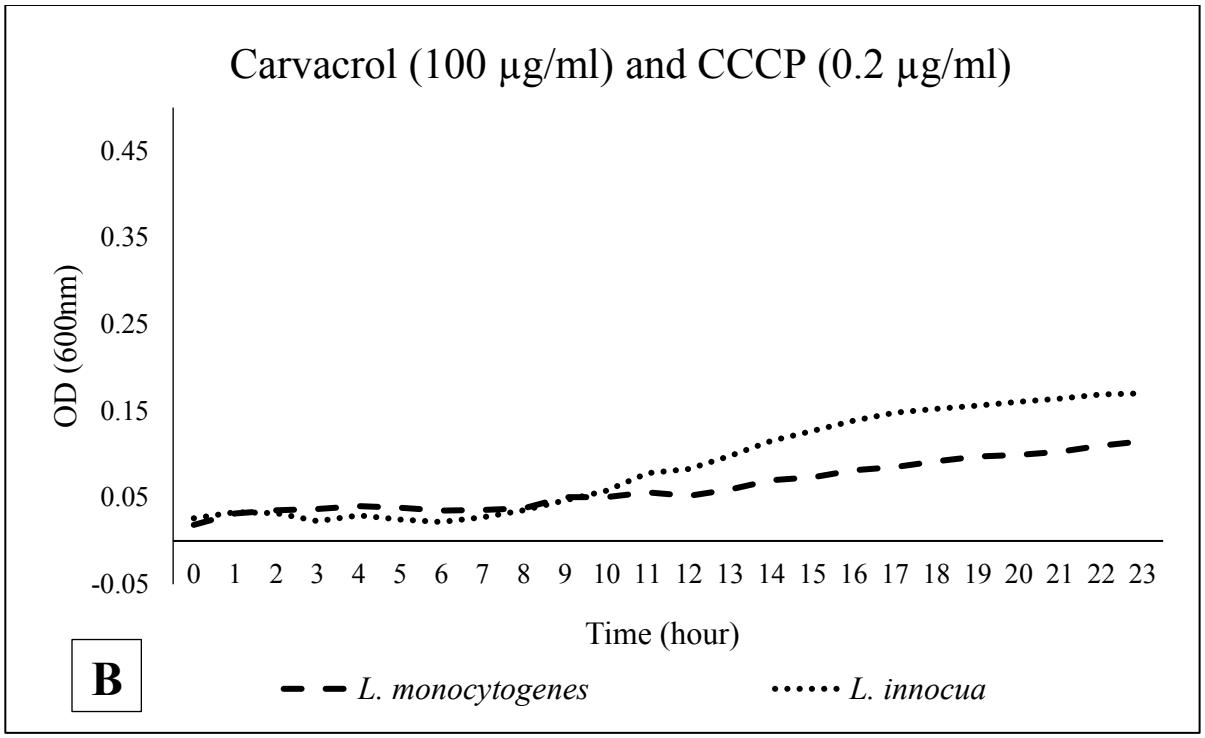
Figure 6: Initial screening (time vs OD_{600nm}) of (A) Cinnamon bark EO at 0.2 µg/ml and 0.05 µg/ml, (B) Lavender EO at 0.2 µg/ml and 0.1 µg/ml, (C) Peppermint EO at 0.2 µg/ml and 0.1µg/ml, (D) Fennel EO at 0.2 µg/ml and 0.1 µg/ml, and (E) Coriander at 0.2 µg/ml and 0.1 µg/ml against *L. monocytogenes* FSL-J1-031 and *L. innocua* 2007-663. Error bars indicate standard deviation. (n=9).

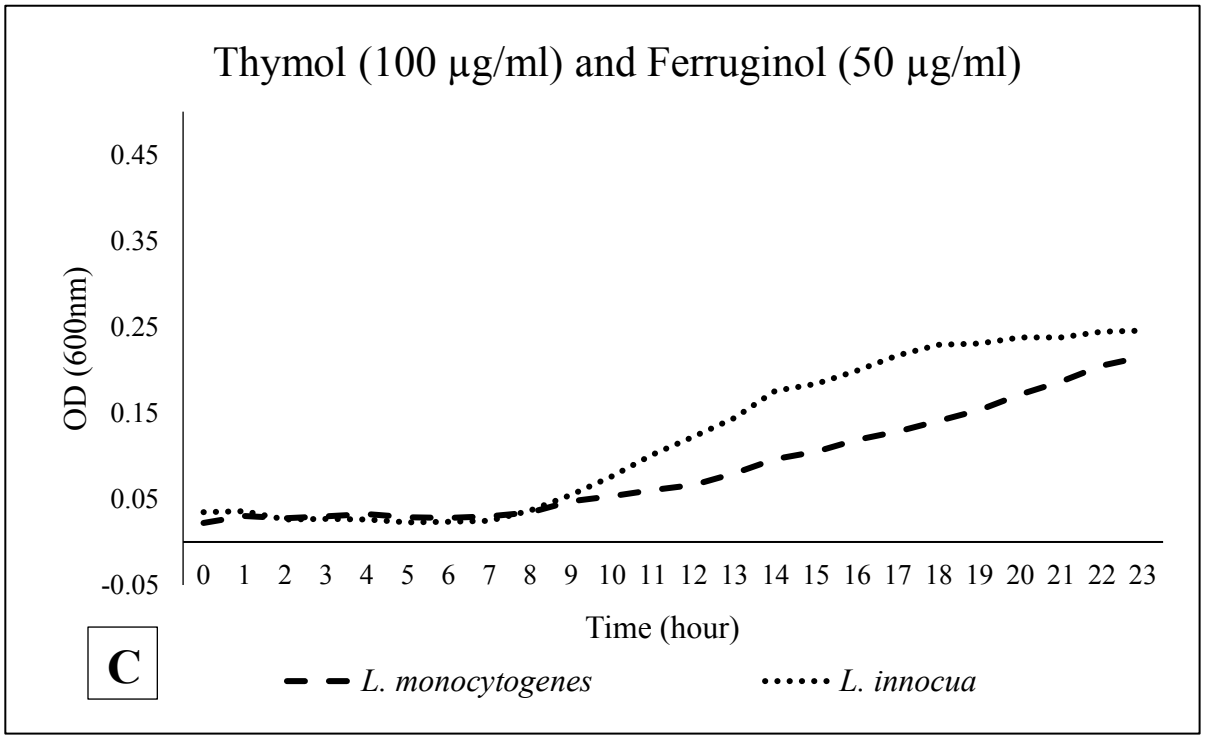
4.2.4 Five Combinations Tested Showed Differential Inhibition between *L. monocytogenes* and *L. innocua*

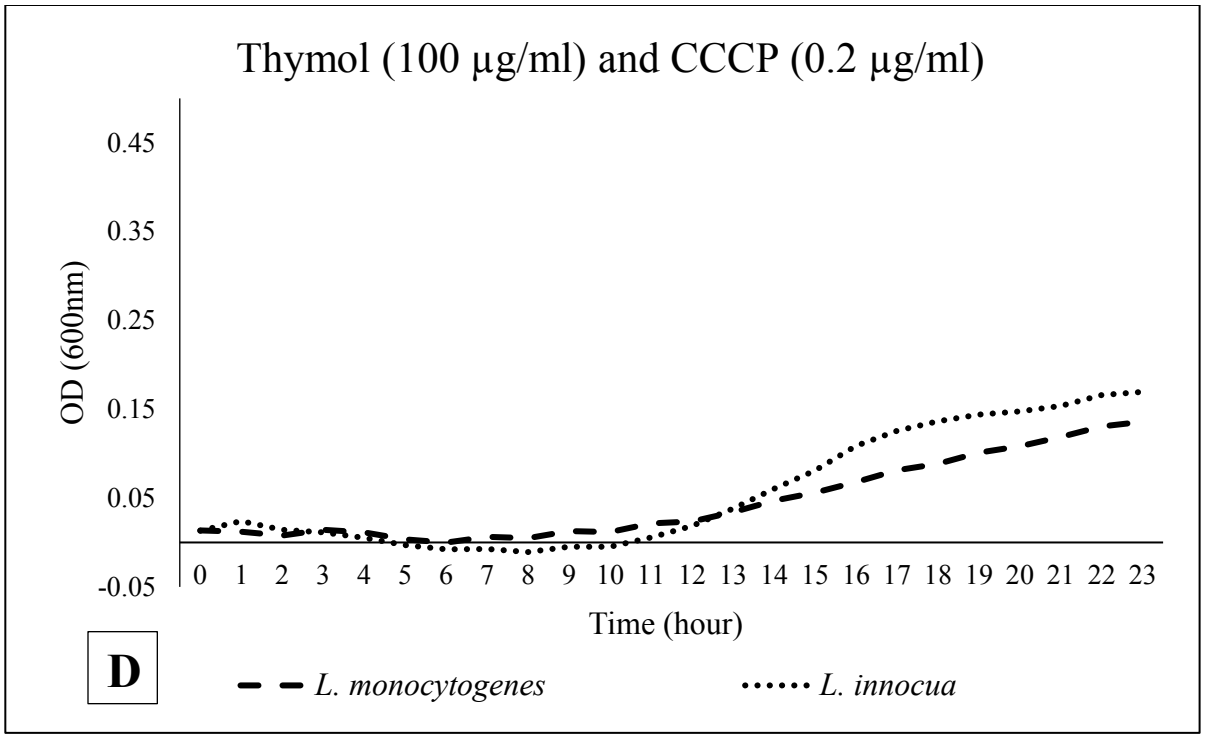
Combinations of phytochemicals were also screened against the *L. monocytogenes* and *L. innocua* strains, with significant differential effects shown for the combinations of carvacrol at 100 µg/ml and ferruginol at 50 µg/ml (Figure 7A), carvacrol at 100 µg/ml and CCCP at 0.2 µg/ml (Figure 7B), thymol at 100 µg/ml and ferruginol at 50 µg/ml (Figure 7C), thymol at 100 µg/ml and CCCP at 0.2 µg/ml (Figure 7D), and cinnamon bark EO at 0.05 µg/ml and ferruginol at 50 µg/ml (Figure 7E).

Initial Screenings of Combinations of Phytochemicals against *L. monocytogenes* and *L. innocua*




Carvacrol (100 µg/ml) and CCCP (0.2 µg/ml)
OD (600nm)
0.45
0.35
0.25
0.15
0.05
-0.05
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (hour)
B
L. monocytogenes
L. innocua


Thymol (100 µg/ml) and Ferruginol (50 µg/ml)
OD (600nm)
0.45
0.35
0.25
0.15
0.05
-0.05
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (hour)
C
L. monocytogenes
L. innocua



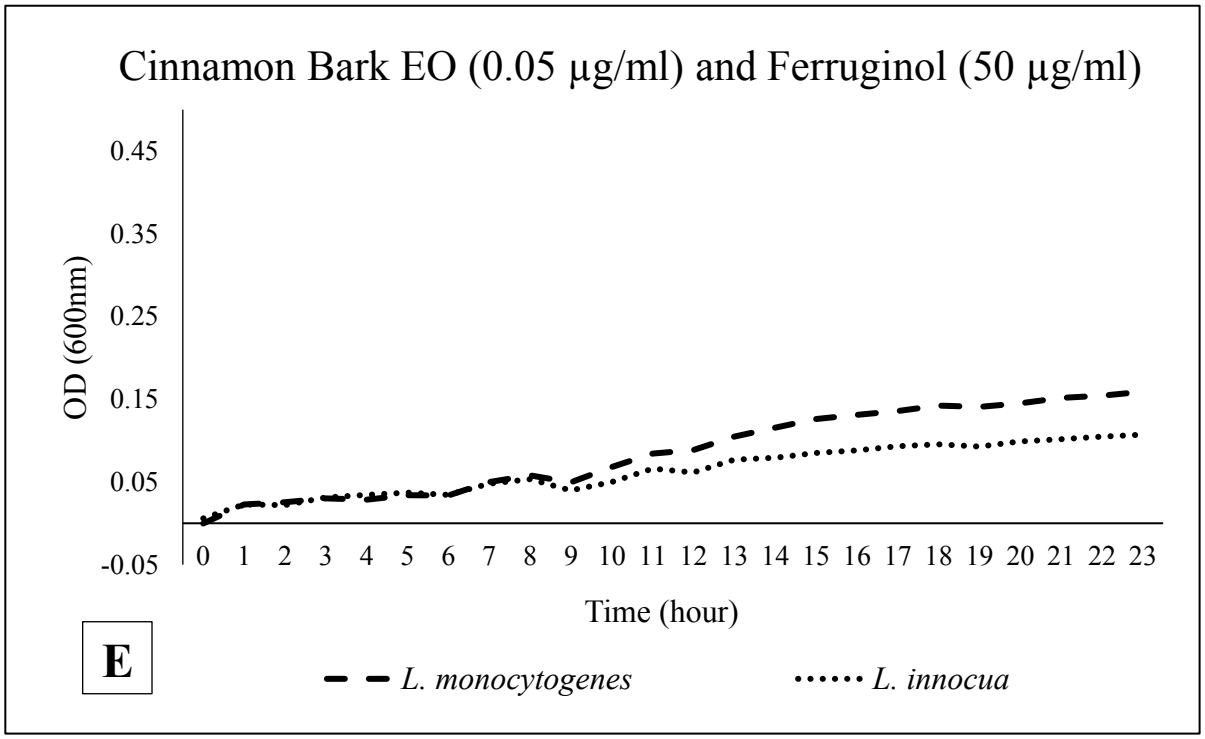


Figure 7: Growth curves (time vs OD_{600}) of the initial screening of the combinations (A) Carvacrol at 100 µg/ml and Ferruginol at 50µg/ml, (B) Carvacrol at 100 µg/ml and CCCP at 0.2 µg/ml, (C) Thymol at 100 µg/ml and Ferruginol at 50 µg/ml, (D) Thymol at 100 µg/ml and

CCCP at 0.2 µg/ml, and (E) Cinnamon Bark EO at 0.05 µg/ml and Ferruginol at 50 µg/ml against *L. monocytogenes* FSL-J1-031 and *L. innocua* 2007-663. (n=9).

Table 6: Summary of compounds or combinations of compounds that favor the growth of *L. monocytogenes* FSL-J1-031 when compared to the growth of *L. innocua* 2007-663.

Compound	**Concentration (µg/ml)**
Eugenol	200
Indole-3-carbinol	50
Ferruginol	200
Silibinin	50
NMP	100
Omeprazole	12.5
Cinnamon bark EO	0.05
Peppermint EO	0.1
Cinnamon bark EO and Ferruginol	0.05 and 50

Table 7: Summary of compounds or combinations of compounds that favor the growth of *L. innocua* 2007-663 when compared to the growth of *L. monocytogenes* FSL-J1-031

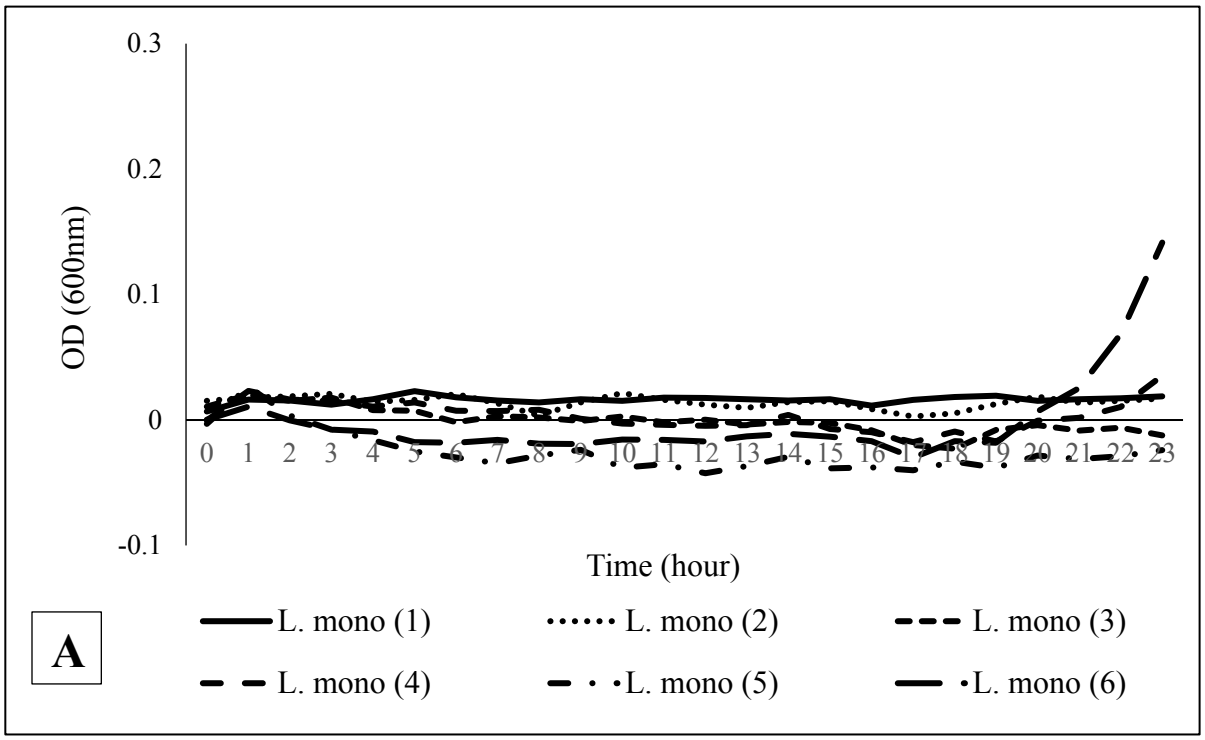
Compound	**Concentration (µg/ml)**
Theaflavin (>90% Purity)	25
Theaflavin (97% Purity)	200
Berberine	200
Lavender EO	0.1
Peppermint EO	0.2

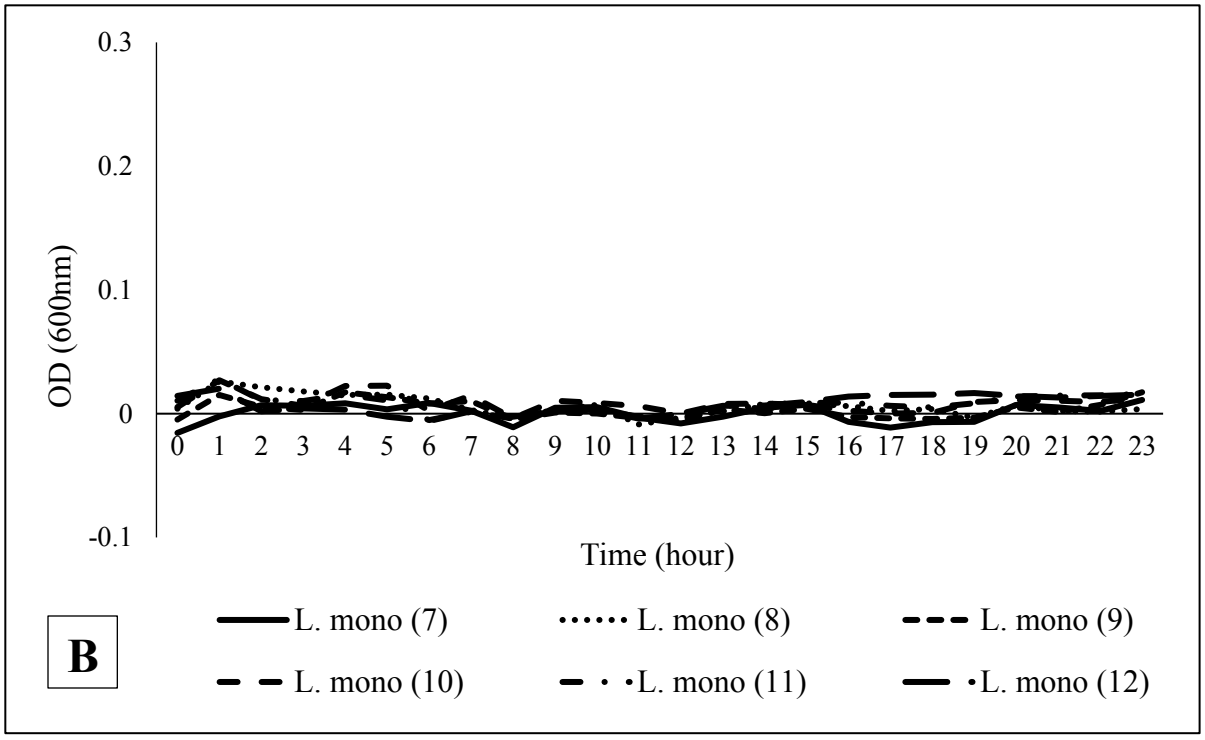
Fennel EO	0.1
Coriander EO	0.1
Carvacrol and Ferruginol	100 and 50
Carvacrol and CCCP	100 and 0.2
Thymol and Ferruginol	100 and 50
Thymol and CCCP	100 and 0.2

4.3 Screening of Phytochemicals against Multiple Strains of *Listeria* Identified Strain-Specific Inhibition Amongst *L. monocytogenes* and *L. innocua*

The individual phytochemicals and combinations thereof showed significant differential effects between *L. monocytogenes* strain FSL-031 and *L. innocua* strain 2007-663 were selected for further tests against a panel of twelve strains each of *L. monocytogenes* and *L. innocua*, to confirm the reproducibility of their effectiveness against a variety of strains. Results for these strains exposed to theaflavin (≥90% Purity) at a concentration of 25 μg/ml are given in Figure 8. For *L. monocytogenes* strains, all were inhibited over the 23 hour incubation period (Figure 8A and 8B), with the exception of L. mono (6) which showed delayed and limited growth after 21 hours. As for *L. innocua*, growth for all but two strains was completely inhibited by the phytochemical. The strain of *L. innocua* denoted as L. inno (11) displayed strong growth indicating full resistance to theaflavin (Figure 8D) at the tested concentration, whilst L. inno (12) was able to briefly recover showing very limited growth after 16 hours (Figure 7D). The effect was indeed bacteriostatic as all strains were able to recover when spotted onto TSA and incubated at 24 hours at 30°C.

Theaflavin (≥90% Purity) against Multiple strains of *Listeria*





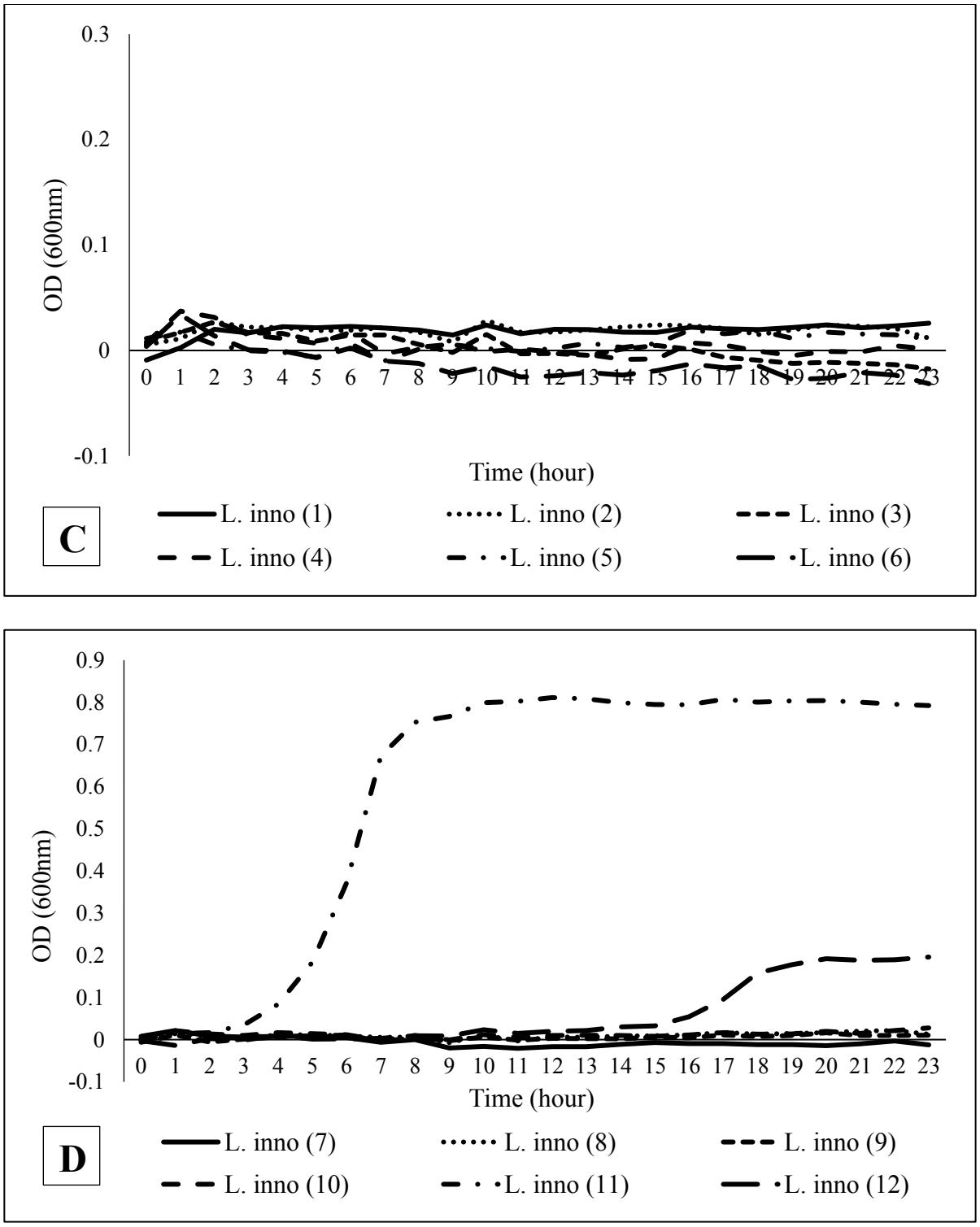
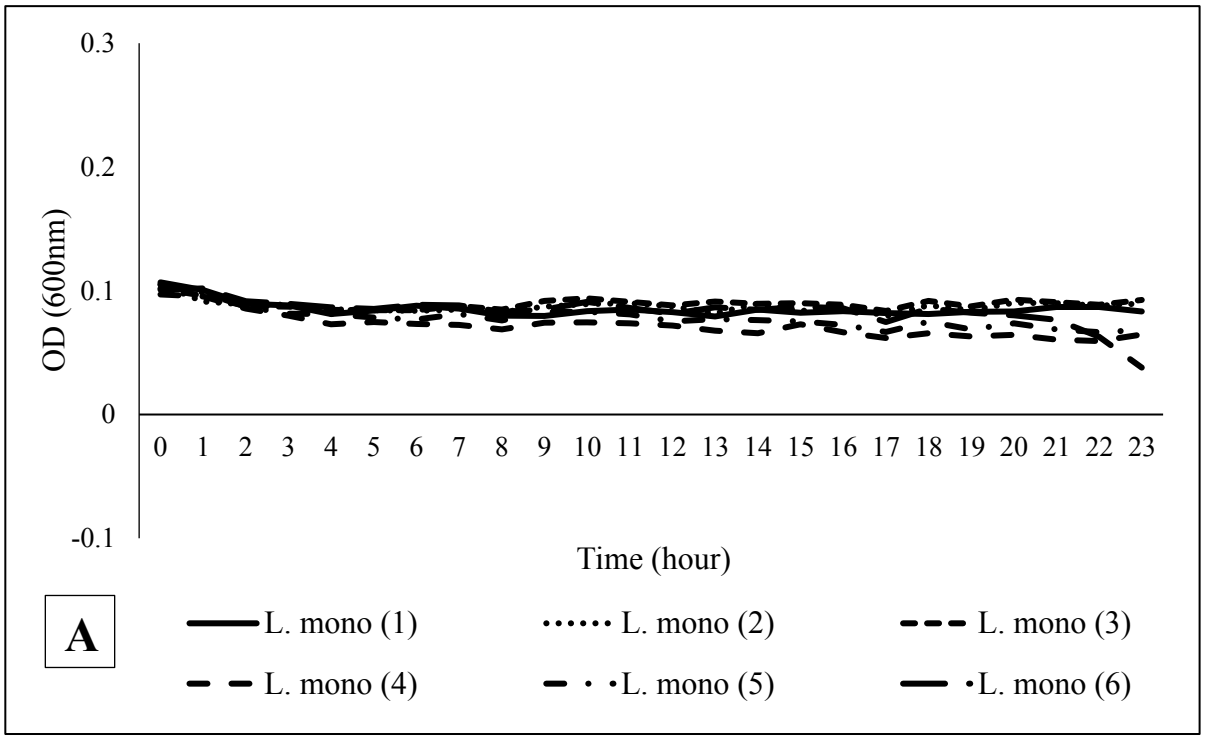
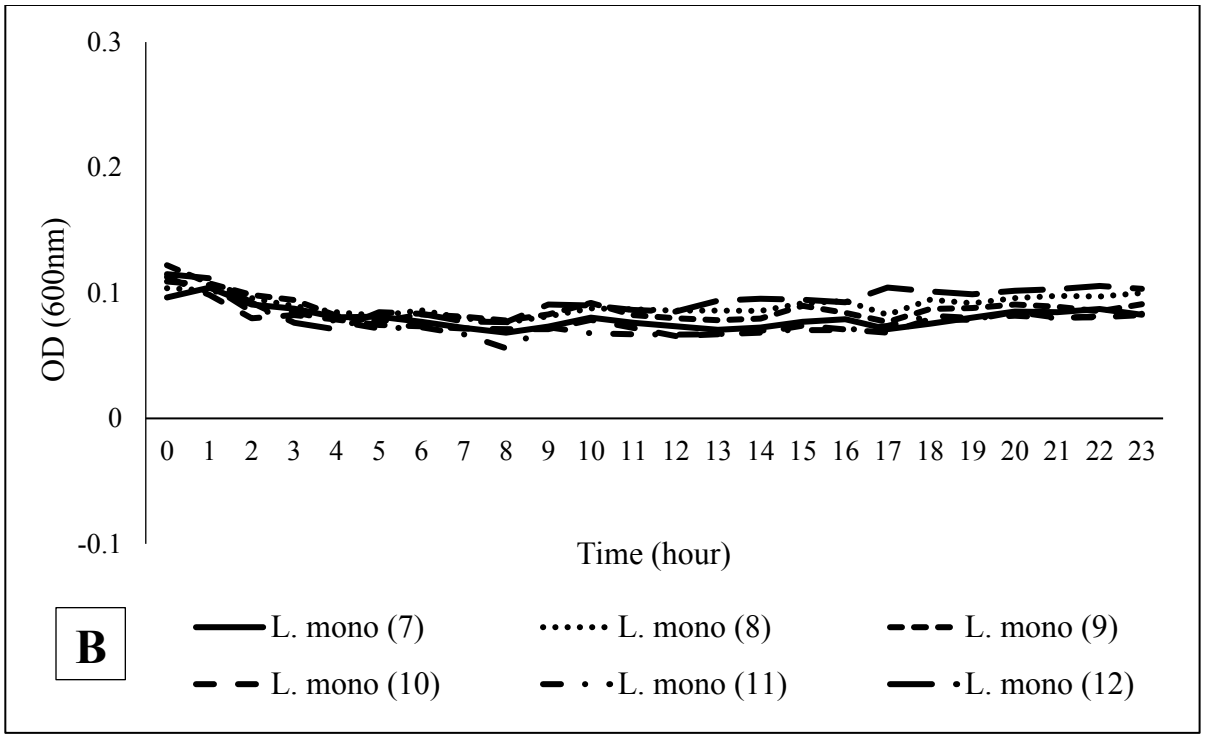


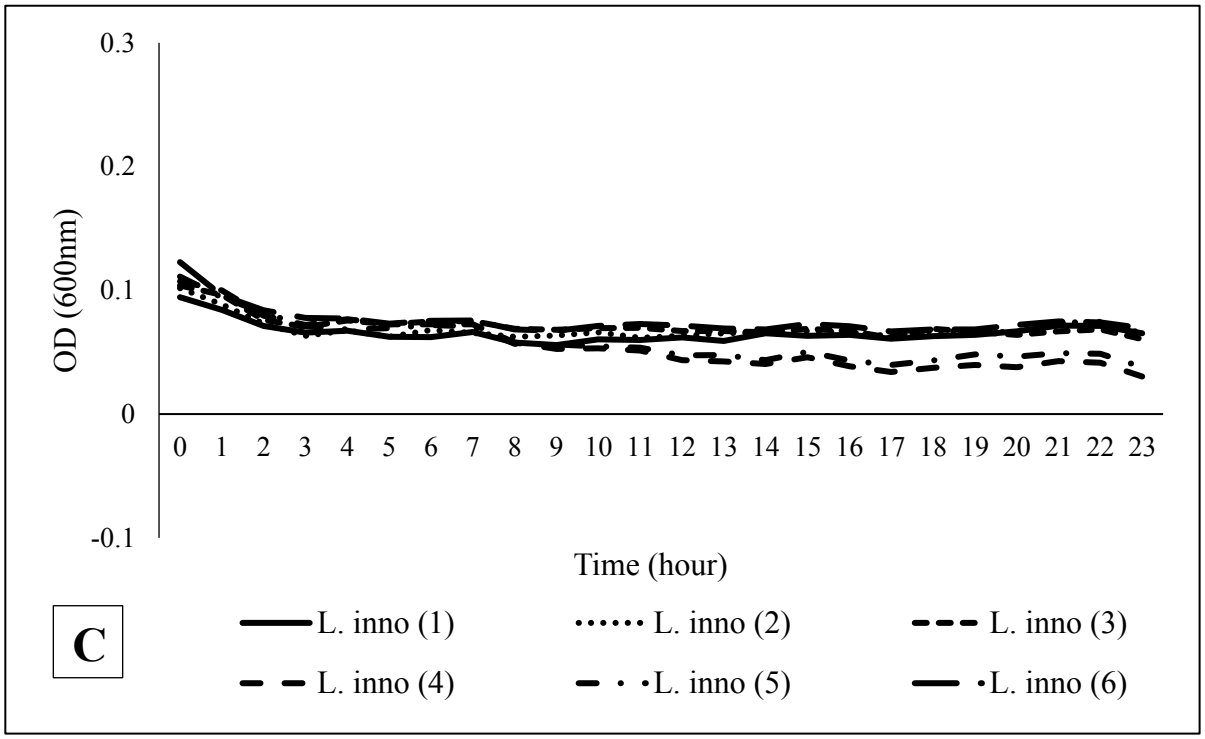
Figure 8: Growth curves (time vs OD_{600}) of Theaflavin (≥90% Purity) at 25 µg/ml against a panel of twelve strains each of *L. monocytogenes* (A and B) and *L. innocua* (C and D). (n=9).

Another of the phytochemical that was subjected to the panel of twenty-four strains of *Listeria* was theaflavin (97% Purity) at 200 µg/ml (Figure 9). The only strain showing growth was L. inno (12) albeit this was severely delayed for 9 hours and plateaued at modest OD values (Figure 9D). All other strains were completely inhibited, with no growth occurring (Figure 9A-D). Again, all strains were able to recover when spotted onto TSA plates and incubated for 24 hours at 30°C, showing that the effect is bacteriostatic.

Theaflavin (97% Purity) against Multiple strains of *Listeria*




0.3
0.2
0.1
0
-0.1
OD (600nm)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (hour)
B
L. mono (7)
L. mono (8)
L. mono (9)
L. mono (10)
L. mono (11)
L. mono (12)


0.3
0.2
0.1
0
-0.1
OD (600nm)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (hour)
C
L. inno (1)
L. inno (2)
L. inno (3)
L. inno (4)
L. inno (5)
L. inno (6)

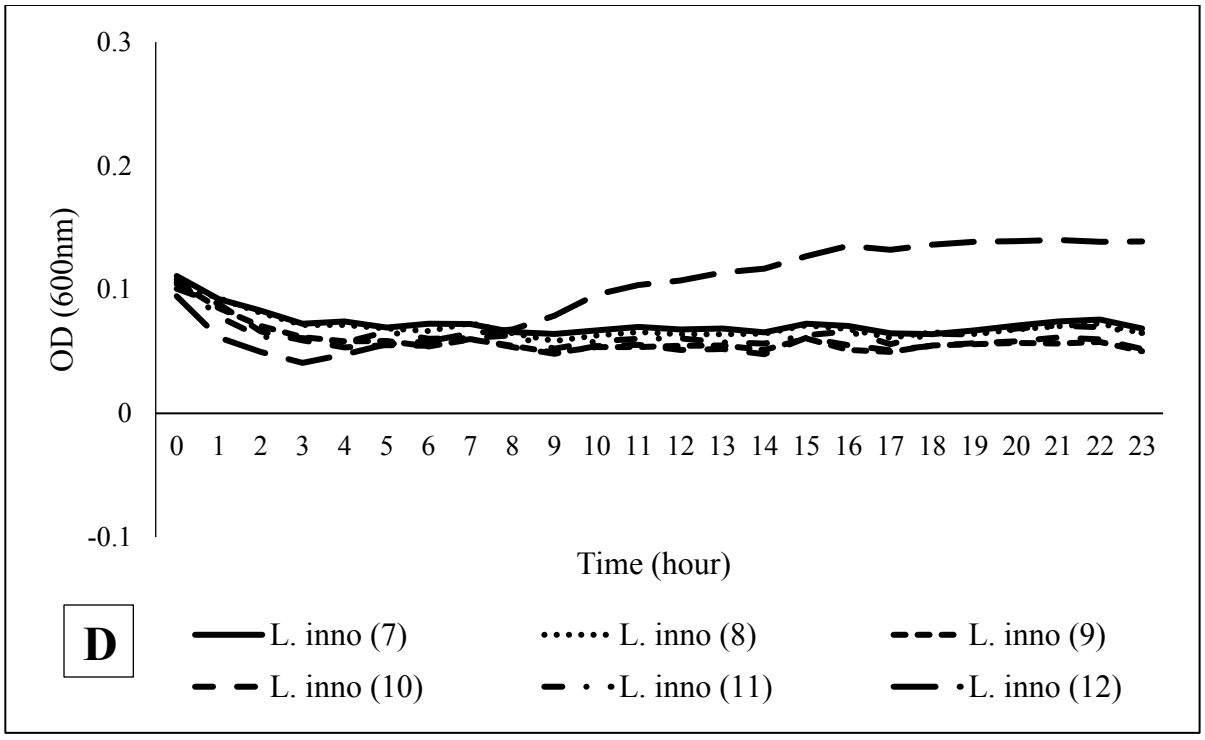
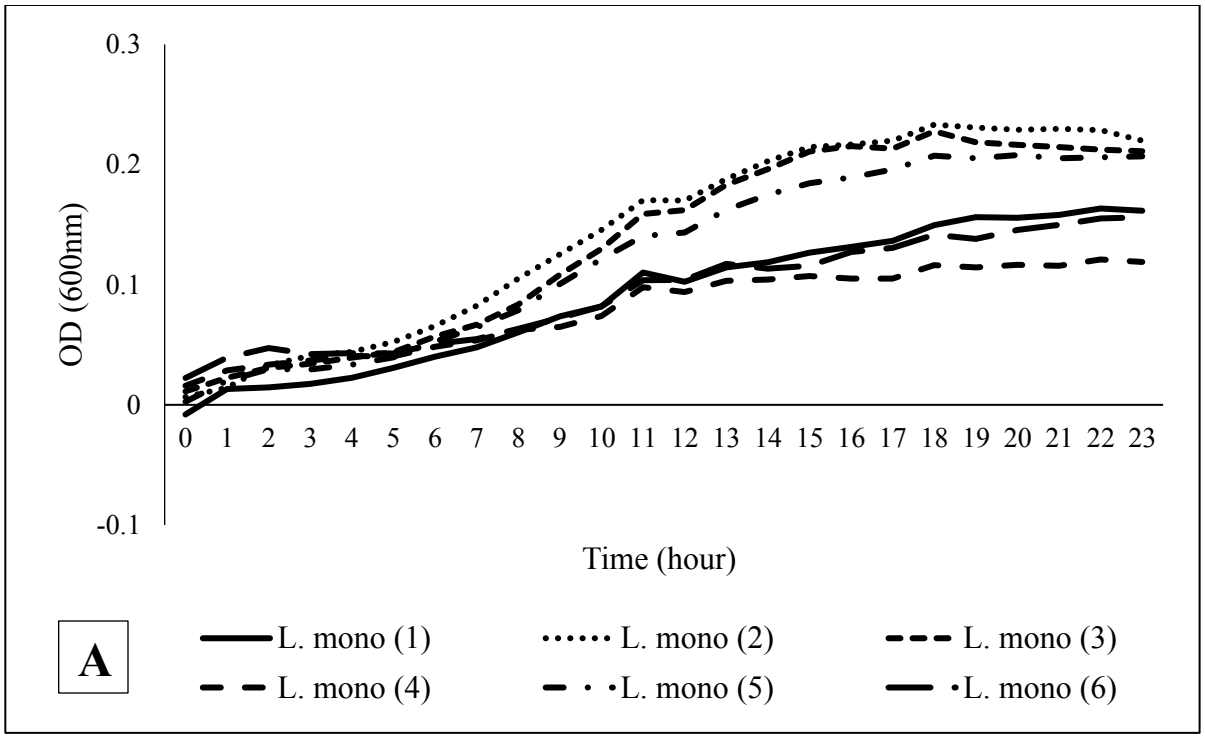
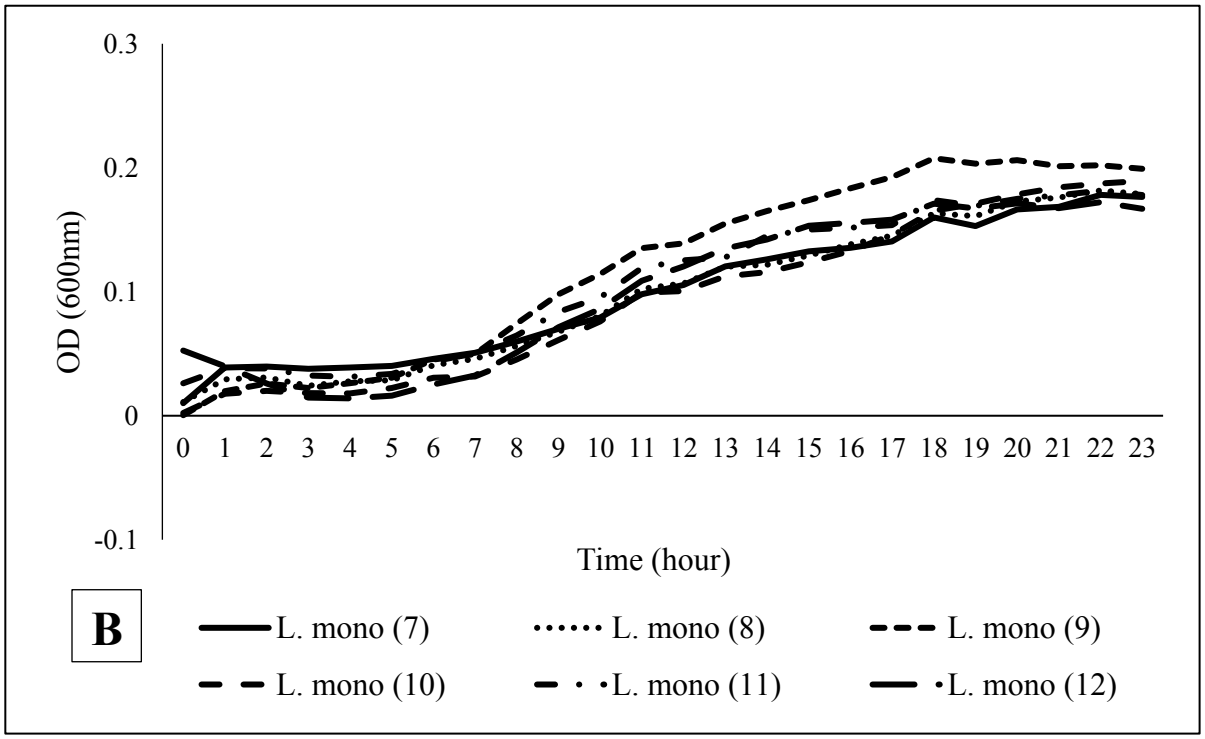


Figure 9: Growth curve (time vs OD_{600nm}) of Theaflavin (97% Purity) at 200 µg/ml against a panel of twelve strains each for *L. monocytogenes* (A and B) and *L. innocua* (C and D). (n=9).

The combination of cinnamon bark EO at 0.05 µg/ml and ferruginol at 50 µg/ml was also tested against the twenty-four strains of *Listeria* (Figure 10). As with the other compounds, *L. monocytogenes* generally displayed greater inhibition than did *L. innocua*, however, four *L. monocytogenes* strains [i.e L. mono (2), L. mono (3), L. mono (5) and L. mono (9)] showed similar growth to the *L. innocua* strains (Figure 10A and B). This is the only phytochemical combination that resulted in significant ($P<0.05$) differences between the twenty-four strains of *Listeria* based on an ANOVA statistical test.

The Combination of Cinnamon Bark EO and Ferruginol against Multiple Strains of *Listeria*


0.3
0.2
0.1
0
-0.1
OD (600nm)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (hour)
A
L. mono (1)
L. mono (2)
L. mono (3)
L. mono (4)
L. mono (5)
L. mono (6)


0.3
0.2
0.1
0
-0.1
OD (600nm)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (hour)
B
L. mono (7)
L. mono (8)
L. mono (9)
L. mono (10)
L. mono (11)
L. mono (12)

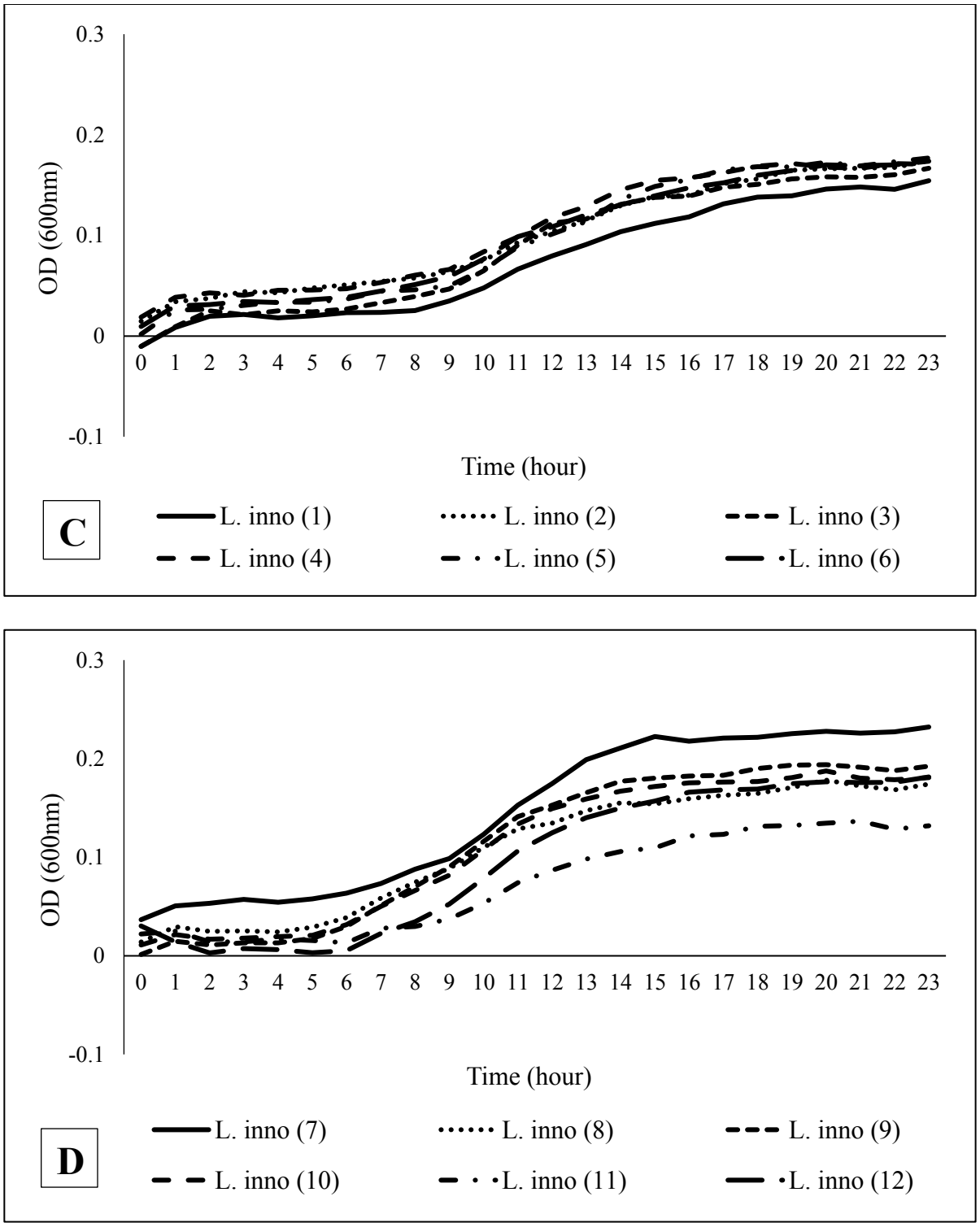
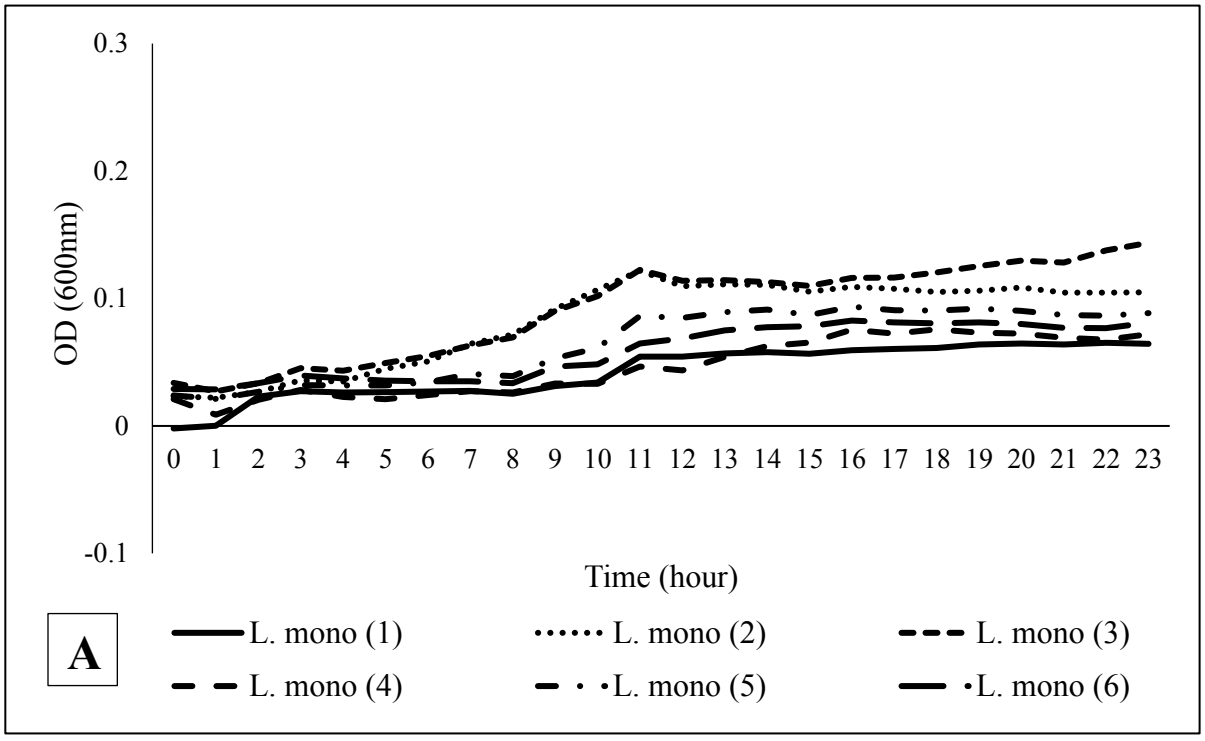
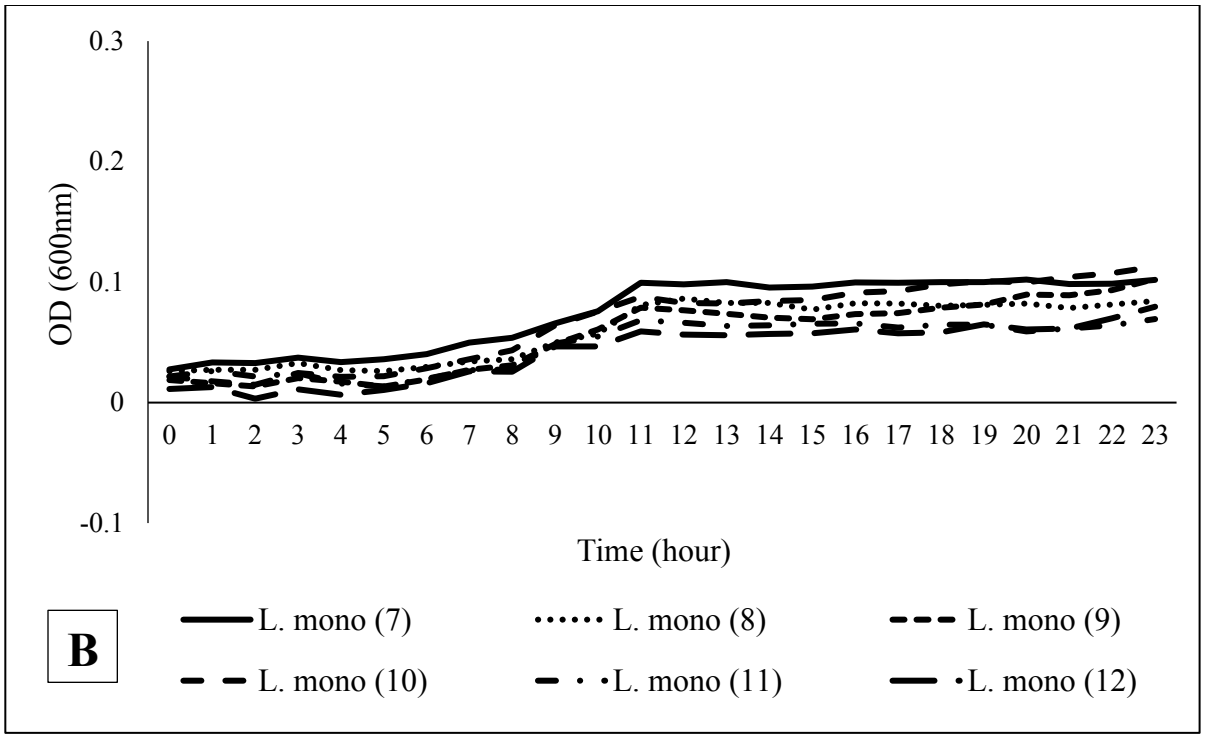


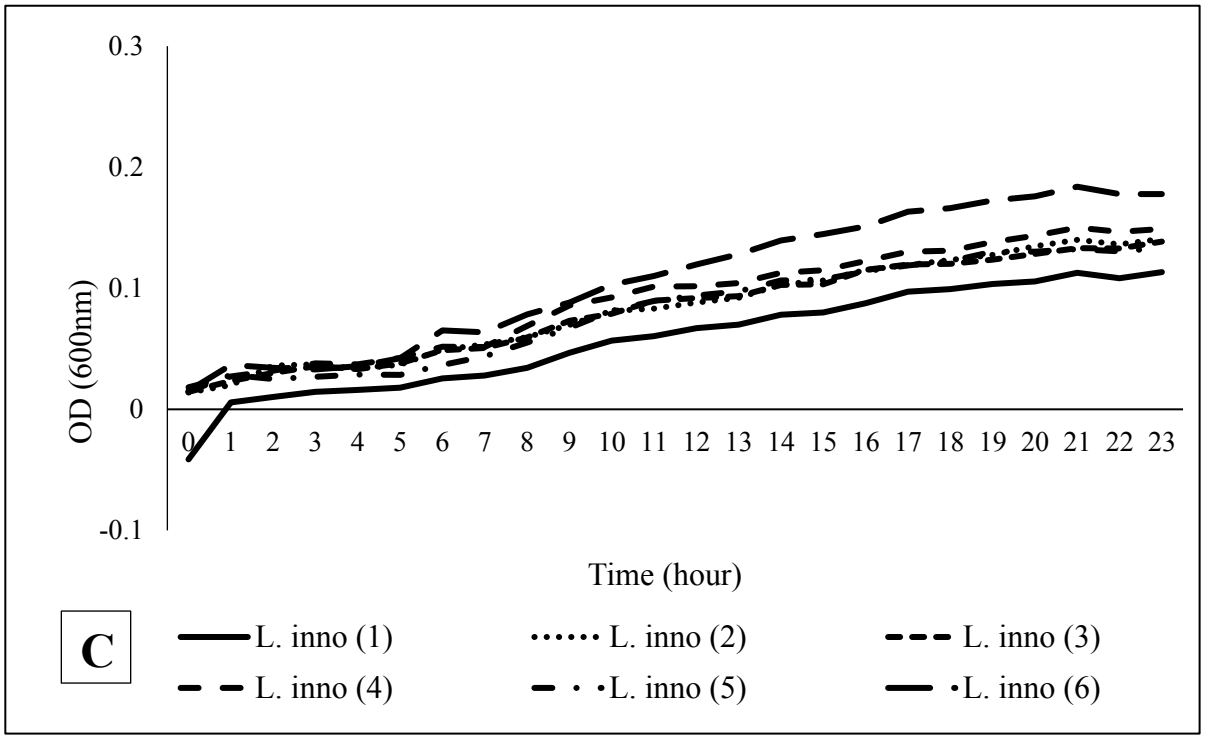
Figure 10: Growth curves for (time vs OD_{600nm}) the combination of Cinnamon bark EO at 0.05 µg/ml and Ferruginol at 50 µg/ml against a panel of twelve strains each of *L. monocytogenes* (A and B) and *L. innocua* (C and D). (n=9).

The final combination of phytochemical utilized against the twenty-four strain panel of *L. monocytogenes* and *L. innocua* was cinnamon bark EO at 0.05 µg/ml and NMP at 25 µg/ml (Figure 11). In general, the *L. monocytogenes* strains were more sensitive to these treatments than the *L. innocua* strains; however, all the strains were inhibited by the combination.

The Combination of Cinnamon Bark EO and NMP against Multiple Strains of *Listeria*




0.3
0.2
0.1
0
-0.1
OD (600nm)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (hour)
B
L. mono (7)
L. mono (8)
L. mono (9)
L. mono (10)
L. mono (11)
L. mono (12)


0.3
0.2
0.1
0
-0.1
OD (600nm)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (hour)
C
L. inno (1)
L. inno (2)
L. inno (3)
L. inno (4)
L. inno (5)
L. inno (6)

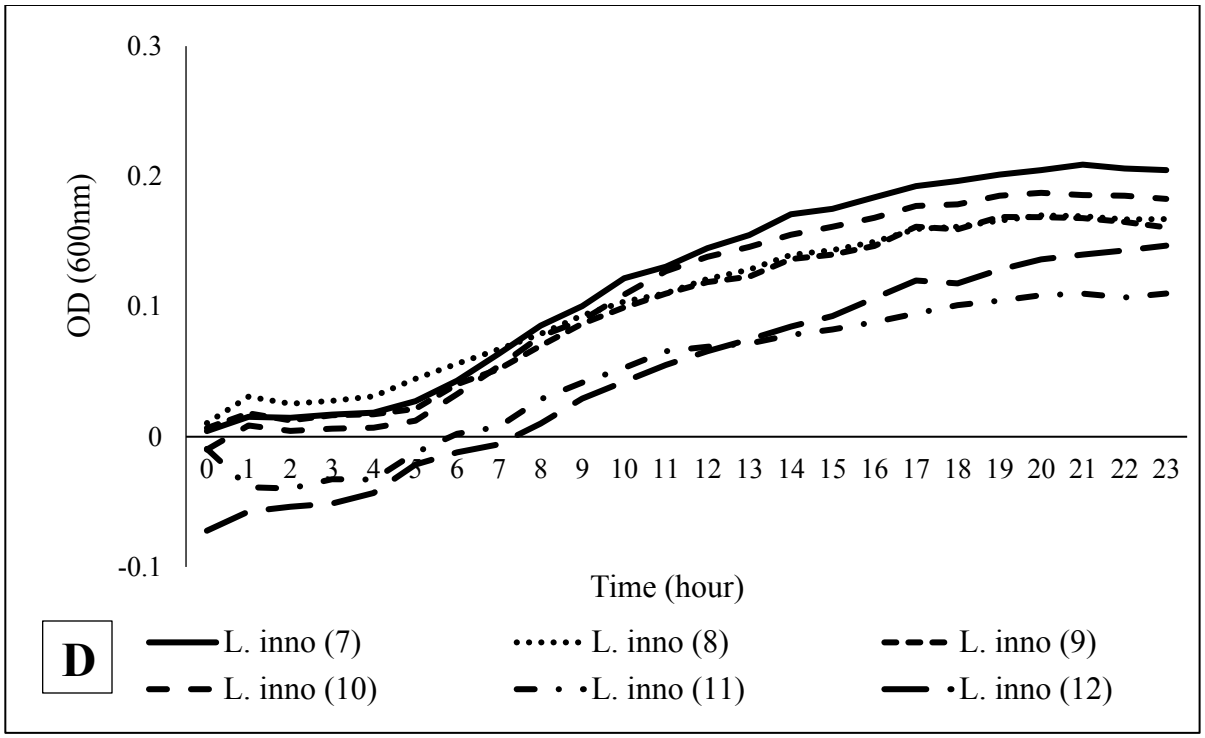


Figure 11: Growth curves (time vs OD_{600nm}) of the combination of Cinnamon bark EO at 0.05 µg/ml and NMP at 25 µg/ml against a panel of twelve strains each of *L. monocytogenes* (A and B) and *L. innocua* (C and D). (n=9).

4.4 Growth Kinetics of Milk Microbiota Biofilm Indicates Optimal Biofilm Formation Occurring at 74 hours

The growth kinetics of the biofilm formed by the microbiota carried in 2% M.F pasteurized milk at 4 °C are shown in Figure 12. The biomass was quantified by the crystal violet binding assay with samples assayed over a 96 hour period. The 2% M.F pasteurized milk sample was determined to have optimal biomass formation at 74 hours. The addition of Cosmic Serum™ to the 2% M.F pasteurized milk (used as the positive control) confirmed that the extra nutrients yielded a higher amount of biofilm biomass when compared to the 2% M.F pasteurized milk sample. The positive control also had optimal biofilm formation occurring at 74 hours. Boiled 2% M.F pasteurized milk (used as the negative control) had consistent biomass throughout the

experiment, indicating that the observed increase in biomass for the 2% M.F pasteurized milk sample is due to bacterial attachment.

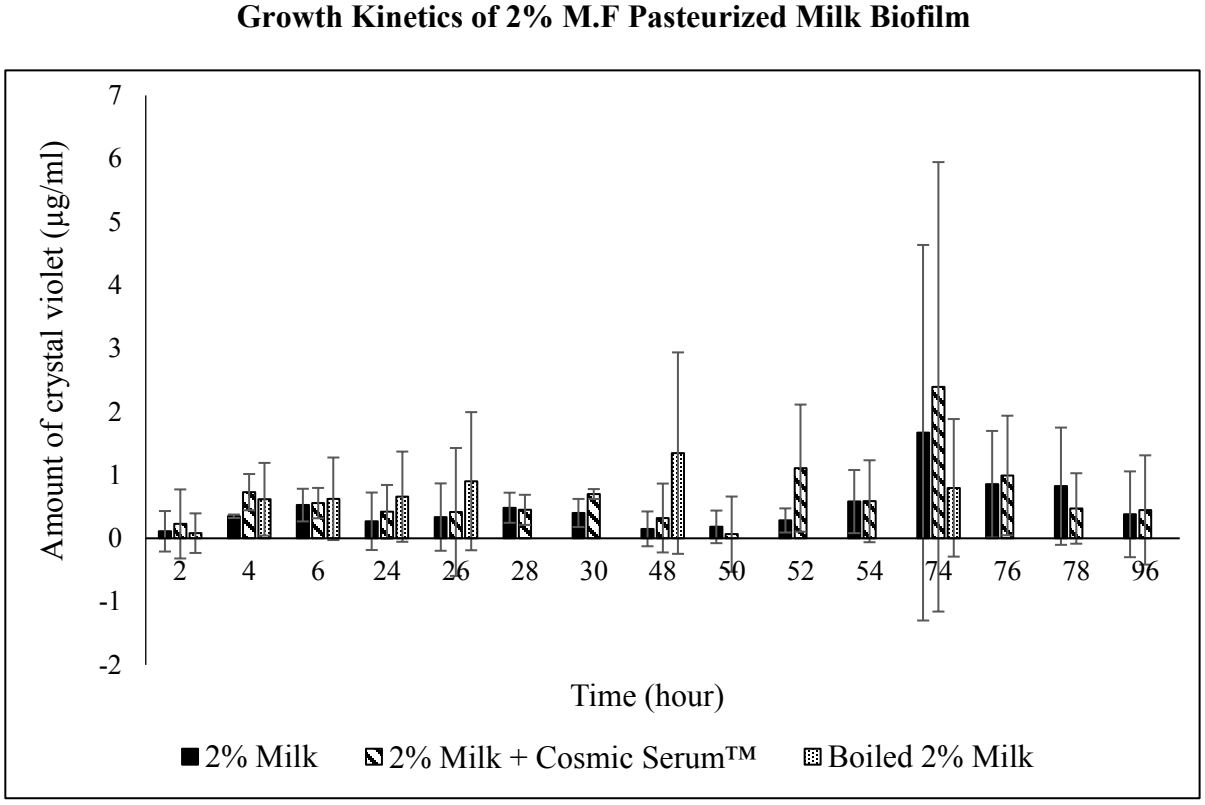


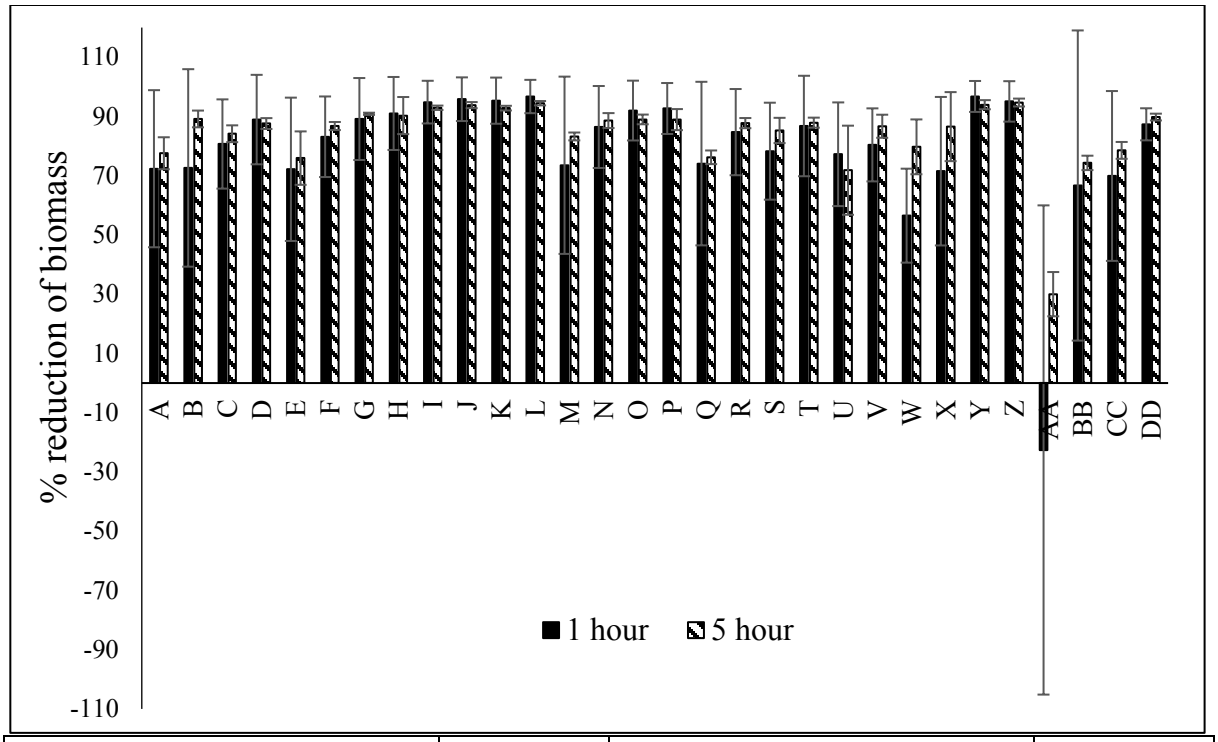
Figure 12: Bar graph (time vs amount of crystal violet) of the crystal violet assay determining the biomass created at 4°C by the microbiota carried in 2% M.F pasteurized milk, 2% M.F pasteurized milk supplemented with Cosmic Serum™ (used as positive control for biofilm formation), and boiled 2% M.F pasteurized milk (used as negative control for biofilm formation). The assay was run on 96-well polystyrene microtiter plates using 200 ul per well. Error bars indicate standard deviation. (n=3).

4.5 Five Phytochemicals Removed Over 89% of Preformed Biofilms in 96-Well Plates

Once the period of optimum biofilm formation was determined, a selection of the secondary plant metabolites was tested for their efficacy to remove preformed biofilms grown in

a 96-well microtiter plate. The preformed biofilms were subjected to the chosen phytochemicals at a high and a low concentration according to values reported in the literature. The biofilms were left for exposure times of 1 and 5 hours at 4°C (Figure 13). The phytochemical that resulted in the largest reduction in biomass were tea tree EO at 6.5% (89% biomass removal at 1 hour exposure, and 91% biomass removal at 5 hour exposure), tea tree EO at 2.6% (91% for 1 hour exposure, 90% for 5-hour exposure), thymol at 305 µg/ml (95% for 1 hour exposure ,93% for 5 hour exposure), thymol at 122 µg/ml (95% for 1 hour exposure, 93% for 5 hour exposure), carvacrol at 1600 µg/ml (95% for 1 hour exposure, 93% for 5 hour exposure), carvacrol at 640 µg/ml (97% for 1 hour exposure, 94% for 5 hour exposure), coriander EO at 6 µl/ml (92% for 1 hour exposure, 90% for 5 hour exposure), coriander EO at 1.2 µl/ml (93% for 1 hour exposure, 89% for 5 hour exposure), eugenol at 305 µg/ml (97% for 1 hour exposure, 94% for 5 hour exposure), and eugenol at 122 µg/ml (95% for 1 hour exposure, 95% for 5 hour exposure). In general, no significant differences between ($P>0.05$) were found between the high and low concentrations of a given chemical and its ability to remove preformed biofilm. The aforementioned phytochemicals were then used in further experiments.

The ability of Phytochemicals to Remove Preformed 2% M.F Pasteurized Milk Biofilm Containing *L. monocytogenes*



Phytochemicals	Legend	Phytochemicals	Legend
Cinnamon bark EO (0.4%)	A	Coriander EO (1.2 µl/ml)	P
Cinnamon Bark EO (0.2%)	B	Anise EO (39 µl/ml)	Q
Thyme EO (4.4%)	C	Anise EO (15 µl/ml)	R
Thyme EO (1.8%)	D	Cassia EO (5.4%)	S
Clove EO (5.2%)	E	Cassia EO (2.2%)	T
Clove EO (2.1%)	F	Fennel EO (39 µl/ml)	U
Tea tree EO (6.5%)	G	Fennel EO (15 µl/ml)	V
Tea tree EO (2.6%)	H	Cinnamaldehyde (1600 µg/ml)	W
Thymol (305 µg/ml)	I	Cinnamaldehyde (640 µg/ml)	X
Thymol (122 µg/ml)	J	Eugenol (305 µg/ml)	Y
Carvacrol (1600 µg/ml)	K	Eugenol (122 µg/ml)	Z
Carvacrol (640 µg/ml)	L	Berberine (1600 µg/ml)	AA
Peppermint EO (24 µl/ml)	M	Berberine (640 µg/ml)	BB
Peppermint EO (9 µl/ml)	N	Oregano EO (6 µl/ml)	CC
Coriander EO (6 µl/ml)	O	Oregano EO (1.2 µl/ml)	DD

Figure 13: Bar graph of the % reduction in preformed 2% M.F pasteurized milk containing *L. monocytogenes* biomass after exposure to plant secondary metabolites at a high and low concentration after 1-hour and 5-hour exposure. Error bars are representative of the standard deviation. (n=9).

4.6 *Listeria monocytogenes* Replicated In Milk Dispensing Pumps

In order to determine if *L. monocytogenes* can harbor and persist in the rotary pumps of the milk dispensing units, viable *L. monocytogenes* numbers in the pumps were monitored over time while the pumps remained at 4°C. As previously described, milk was replaced every 3 days after a sample was taken. The results of those samples were below detection limit and considered 0 CFU/ml on day 0, on day 3 5.25×10^1 CFU/ml on day 6 2.45×10^3 CFU/ml and finally, 6.141×10^4 CFU/ml on day 9 (Figure 14) indicating proliferation of *L. monocytogenes* in the system over the nine days.

Enumeration of *L. monocytogenes* in Dispensing Pumps and Attached Silicon Tubing

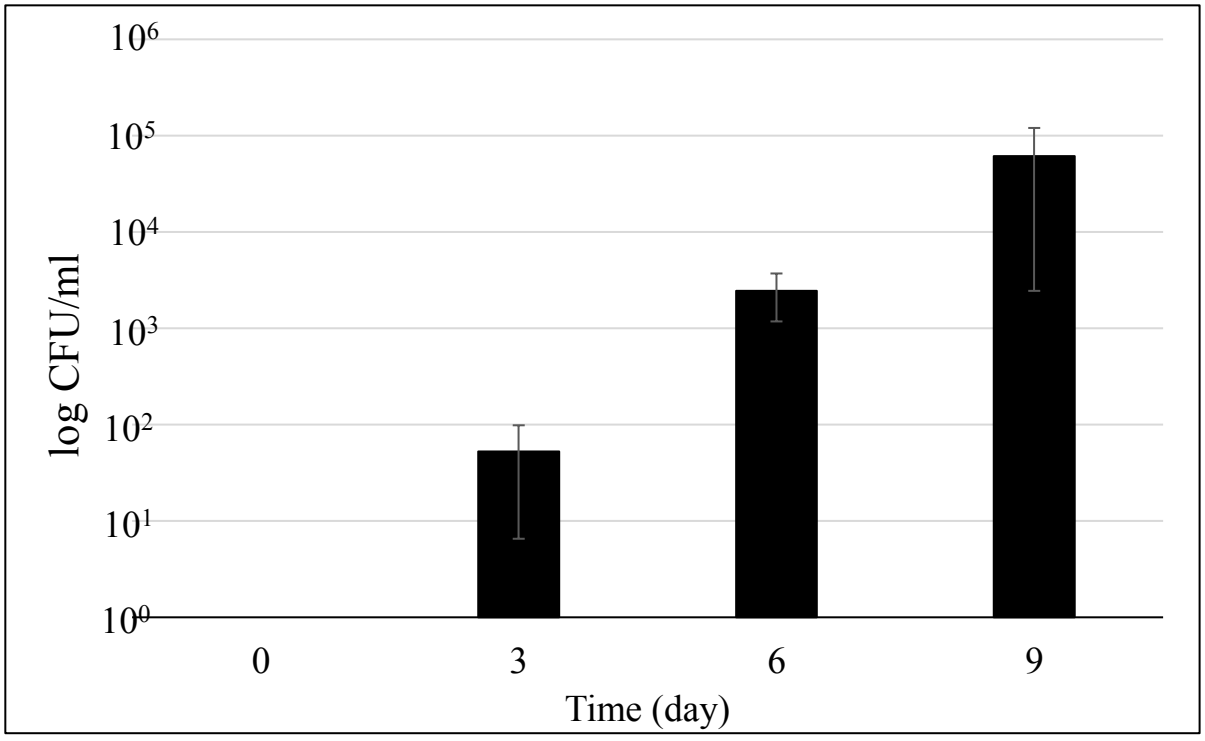


Figure 14: Enumeration of *L. monocytogenes* in 2% M.F pasteurized milk, that was circulated in the pumps of milk dispensing units over 9 days while being incubated at 4°C. Error bars represent standard deviation. (n=3).

4.7 The Ability of Phytochemicals to Remove Preformed Biofilms is Decreased in Dispensing Pumps

Utilizing the phytochemicals identified above (section 4.5), preformed biofilms in the pumps were subjected to the high concentration of five selected compounds (i.e tea tree EO, thymol, carvacrol, coriander EO, eugenol) for 1 hour (Figure 15). None of the plant secondary metabolites tested were able to substantially remove the preformed biofilms in the pumps. Conversely, tea tree EO actually increased the biofilm biomass by 13% and coriander EO increased biomass by 24%. The remaining compounds were found to marginally reduce biomass with thymol reducing by 17.0%, carvacrol by 8.5% and eugenol by 4.8%.

Removal of Preformed 2% M.F Pasteurized Milk Biofilm in Dispensing Pumps and Attached SiliconTubing with Selected Phytochemicals

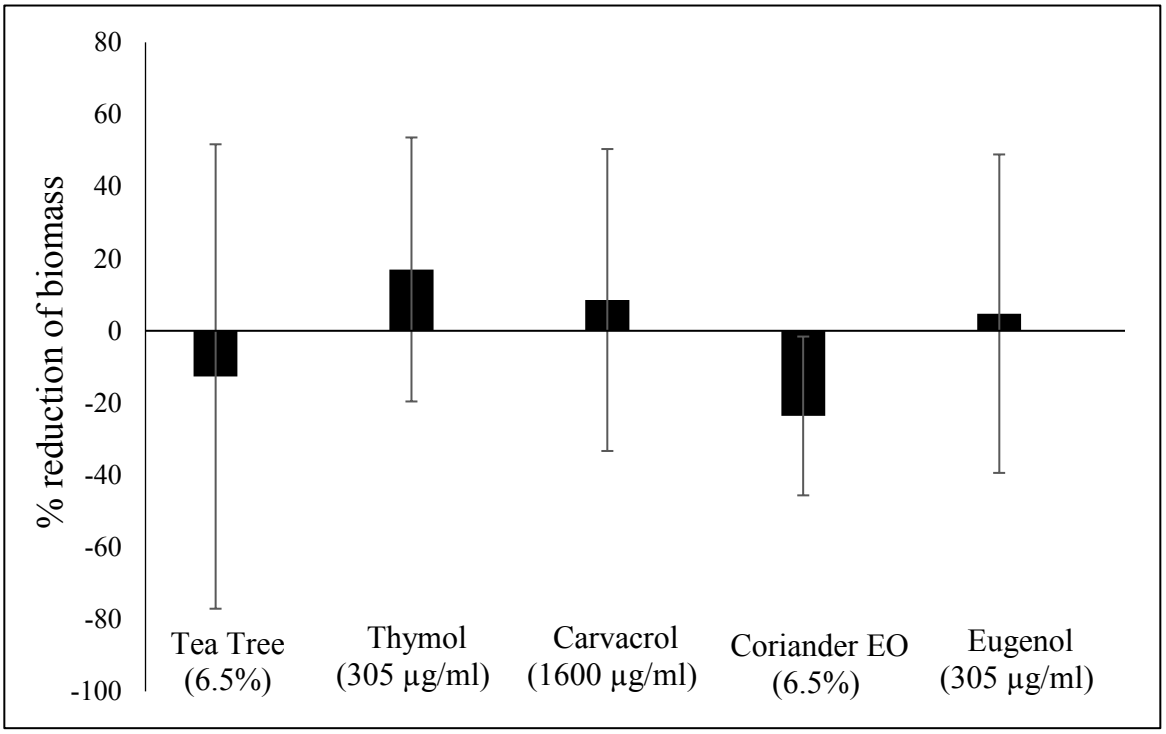


Figure 15: Removal of preformed biomass in milk dispensing pumps by tea tree EO at 6.5%, thymol at 305 µg/ml, carvacrol at 1600 µg/ml, coriander EO at 6.0% and eugenol at 305 µg/ml after 1-hour exposure. Error bars represent standard deviation. (n=3).

4.8 The ability of Thymol and Carvacrol to Remove Preformed Biomass in Dispensing Unit Pumps is Limited, Even When Mechanical Disruption is Used

As none of the phytochemicals were unable to significantly reduce the amount of preformed biofilms in the following a single exposure alone, thymol and carvacrol were chosen to determine if treatments could be effective in concert with the addition of the mechanical action in the form of the pumps circulating the compounds for 15 minutes; followed by a 15 minutes rest period and then a final circulation of 15 minutes (Figure 16). Although the phytochemicals were unable to significantly reduce the preformed biomass, they did, however, remove a greater amount of biomass (thymol removing 32% and carvacrol removing 23%) with mechanical action than when the compounds were left static in the pumps. It was noted in the previous experiment (with the compounds siting in the pumps) that carvacrol interacted negatively with the materials of the pumps, so a lower concentration was utilized for the experiment with mechanical action.

Removal of Preformed 2% M.F Pasteurized Milk Biofilm in Dispensing Pumps and Attached Silicon Tubing with Thymol or Carvacrol with the Addition of Mechanical Action

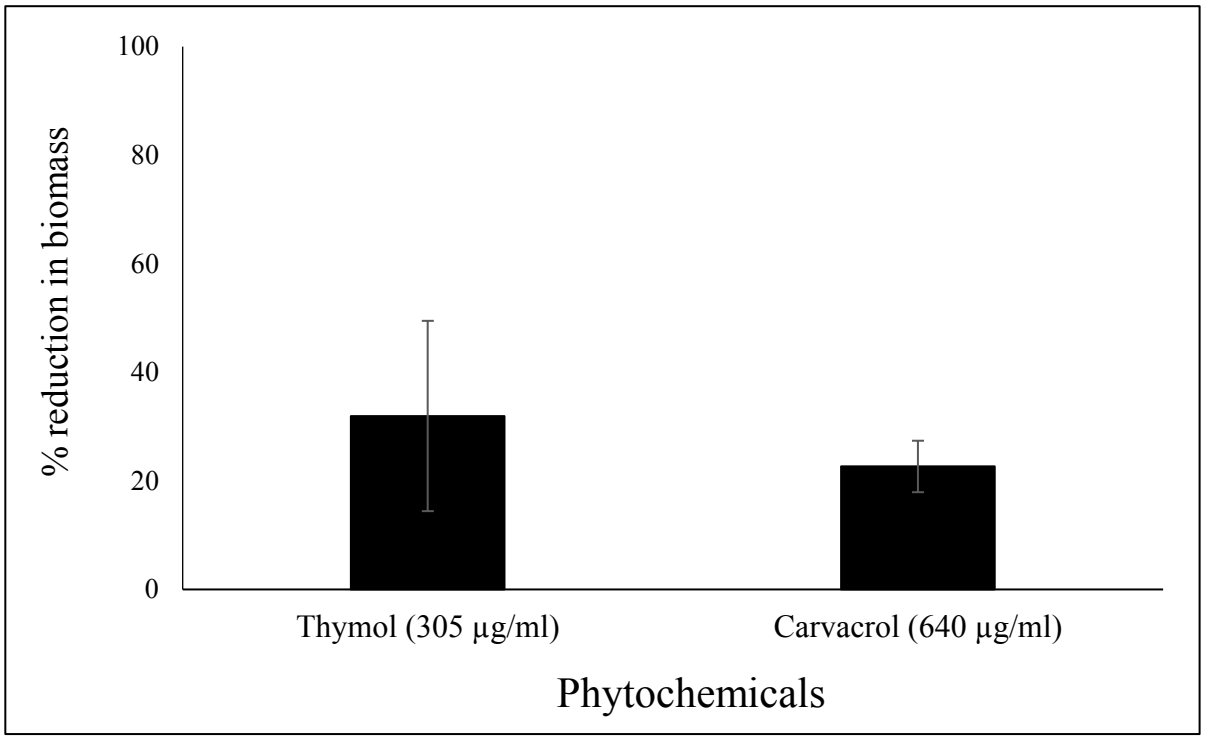


Figure 16: Reduction of biomass in milk dispensing pumps when exposed to thymol at 305 µg/ml and carvacrol at 640 µg/ml with 30 minutes of circulating the pumps and 15 minutes of siting. Error bars represent standard deviation. (n=3).

CHAPTER 5 DISCUSSION

5.1 Minimum Inhibitory Concentration (MIC)

The aim of the first part of this study was to determine phytochemicals that had greater inhibition on the growth of *L. innocua* than on *L. monocytogenes.* Therefore the MICs of the 28 phytochemicals were determined against a single *L. monocytogenes* strain (i.e. Lm-F-117) and found to range from 0.075 µg/ml to 200 µg/ml. This is an accordance with other studies where the MIC values of various phytochemicals against other Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus megaterium*, were reported to be in the range of 100 µg/ml to 250 µg/ml (Abreau et al, 2012). As a group, the *L. monocytogenes* strains were generally more sensitive to the efflux pump inhibitors used in this study than they were to the general antimicrobials which were apparent by the lower MIC values. Additionally, these efflux pump inhibitors were utilized in UVM1 broth which contains the toxic dye, acriflavine, and the antibiotic, nalidixic acid. Both of these compounds are expelled by efflux pumps in *L. monocytogenes*, (Carvalheira et al, 2010) lending to its natural resistance and therefore use of the dye and antibiotic in selective media. However, in the current study the addition of the efflux pump inhibitors in the UVM1 broth, resulted in *L. monocytogenes* becoming susceptible to these compounds. The third group of phytochemical tested, the essential oils, had inhibitory effects at lower concentrations against *L. monocytogenes* Lm-F-117 similar to the efflux pump inhibitors.

5.2 Initial Screening of Phytochemicals against *L. monocytogenes* and *L. innocua*

To determine if the phytochemcials could potentially differentially select between *L. monocytogenes* over *L. innocua,* the phytochemicals were screened against the strain of *L. monocytogenes* (FSL-J1-031) and the strain of *L. innocua* (2007-663) at a high and low concentration. Many compounds showed a differential effect between the two *Listeria* species

with more favoring *L. innocua* growth over *L. monocytogenes*. However, eugenol was the only phytochemical in this group where *L. monocytogenes* showed better growth over *L. innocua.* This phytochemical should be further studied to determine how it is able to differentially inhibit the two species of *Listeria.* The other phytochemicals with general antimicrobial properties were found to favor *L. innocua* growth over *L. monocytogenes*.

The efflux pump inhibitors in UVM1 broth that resulted in significantly differentiated growth between the *L. monocytogenes* strain and the *L. innocua* strain were ferruginol at 200 µg/ml, silibinin at 50 µg/ml, NMP at 100 µg/ml, and omeprazole at 12.5 µg/ml. In the presence of each of these phytochemicals at their stated concentrations, *L. monocytogenes* displayed better growth over *L. innocua.* Therefore, in relation to the aim of this research, these chemicals could have potential as a UVM1 additive for the selection of *L. monocytogenes* over other listeriae. These results support Langille (2015) findings that *L. monocytogenes* utilized a MATE efflux pump to expel acriflavine rather than a MFS efflux pump like *L. innocua.*

When essential oils were screened, cinnamon bark EO and peppermint EO were found to favor *L. monocytogenes* growth over that of the *L. innocua* strain. The converse was true for lavender EO, fennel EO, and coriander EO as the *L. innocua* strain was able to grow better in their presence than the *L.monocytogenes* strain. Further analysis of cinnamon bark EO and peppermint EO to determine their species-specific inhibitory mechanism is needed.

To examine possible synergistic effects, combinations of phytochemicals with general antimicrobial properties and those known to be efflux pump inhibitors were screened in UVM1 broth against the same two *Listeria* spp. strains. All combinations except the cinnamon bark EO with ferruginol resulted in superior growth for *L. innocua* over *L. monocytogenes*; however, the overall trend for these combinations was that they greatly suppressed *Listeria* growth even with

the concentrations lowered for each compound. This indicates that the combinations worked synergistically, perhaps simultaneously affecting different efflux pumps.

These results show that phytochemicals can differentiate between *L. monocytogenes* and *L. innocua* at varying degrees with *L. innocua* still having the advantage in most scenarios. However, since different efflux pump inhibitors were identified where the *L. monocytogenes* strain displayed preferential growth over the *L. innocua* strain this further supports Langille's (2015) accounts that *L. monocytogenes* utilized a different efflux pump(s) to expel acriflavine than *L. innocua* when growing in Fraser broth. Further investigation into the mechanism of action is required to understand how to better select for *L. monocytogenes* for its detection within food products or environmental samples.

5.3 Screening Phytochemicals against Multiple Strains of *Listeria*

The phytochemicals that showed a significant differential effect between *L. monocytogenes* strain FSL-J1-031 and *L. innocua* strain 2007-663 in the initial trials were further screened against twelve strains each of *L. monocytogenes* and *L. innocua*. Theaflavin (≥90% Purity) at the concentration of 25 µg/ml in BHI broth was screened first and the only strain showing complete resistance an *L. innocua* that had been denoted as L. inno (11) for the purpose of these broader screening trials. This was the same strain (i.e 2007-663) utilized in the initial screening comparing the effects of all phytochemicals on a single *L. monocytogenes* strain and a single *L. innocua* strain. The observed unincumbered growth of this *L. innocua* strain was consistent with that displayed during the initial trial. Additionally, one other strain of *L. innocua*, denoted L. inno (12), did show limited growth after a 16-hour lag phase. To further investigate the observed growth kinetics of the L.inno (11) strain, Theaflavin (97% Purity) was then used to screen all twenty-four strains of *Listeria*. This time L. inno (12) was able to grow after a

substantial lag phase recovering at hour 9, albeit its growth was still severely limited therafter. Interestingly, all other strains, including L.inno (11), were completely inhibited by the higher purity compound at its tested concentration. The differing MIC values and inhibitory effects between the two theaflavin compounds on the tested *Listeria* strains may be due to their purity levels, where the Sigma-Aldrich sample of theaflavin had a purity of ≥90.0% and the ChromaDex sample had a purity of 97.0%. Further research is needed to verify the exact cause of differences between the two theaflavin compounds.

As neither theaflavin compound resulted in preferential growth of *L. monocytogenes* over *L. innocua,* the combination of cinnamon bark EO and ferruginol in UVM1 broth was tested against the twenty-four strain panel of *Listeria* spp. This combination resulted in growth differences between the twenty-four *Listeria* species. In this case, *L. monocytogenes* displayed greater sensitivity to the phytochemical combination than *L. innocua*; however, growth of the *L. monocytogenes* strains denoted L. mono (2), L. mono (3), L. mono (5) and L. mono (9) was similar to that of the *L. innocua* strains. These results indicate that the inhibitory effect observed is strain-specific instead of species-specific. Possible reasons for the strong strain-specific effect of phytochemicals, in general, could be speculated to be the presence of different surface proteins on the cell membrane (including different efflux pump systems), as well as a different cell membrane lipid composition. Since, antimicrobial phytochemicals are known to affect the cell membrane (add reference), it is possible that slight changes in membrane composition would result in significant variations in MIC values, but these hypotheses would require experimental validation.

5.4 Determining Growth Kinetics of Milk Microbiota Biofilm

To understand how the microbiota of 2% M.F pasteurized milk behaves during storage 4°C, the growth kinetics of biofilm development was investigated. Using the crystal violet staining assay to assess the biofilms, the 2% M.F pasteurized milk sample was determined to have maximum biomass formation at 74 hours. For these assays, 2% M.F pasteurized milk fortified with the addition of Cosmic Serum™ acted as the positive control as the addition of the bovine serum increased the protein content in the milk. Generally, the positive control had increased biomass over the 2% M.F pasteurized milk, indicating that the Cosmic Serum™ increased the protein content in the milk resulted in great microbial biofilm activity. Similar to the 2% M.F pasteurized milk sample the maximum biomass of the positive control also occurred at 74 hours. Boiled 2% M.F pasteurized milk acted as the negative control as the process of boiling the milk limited the microbiota likely to grow in the milk. The boiled 2% M.F pasteurized milk had similar biomass throughout the experiment duration ensuring that the increase in biomass for the 2% M.F pasteurized milk sample is due to bacterial growth.

Beena et al (2011) assessed the microbiological quality of milk that was processed and pasteurized through an organized dairy plant. They found that the predominant bacterial species found in the pasteurized milk was, *Pseudomonas aeruginosa*, which should have been eradicated through the pasteurization process. Therefore, its presence likely indicated post-pasteurization contamination of the product. It is possible that these bacteria could have been disseminated from an existing biofilm somewhere in the facility. Yuan et al (2019) determined that the *Pseudomonas* strains present in milk could adhere to various surfaces found within the dairy industry and form varying degrees of biofilm. When Sharma and Annand (2002) evaluated biofilms in the post-pasteurization line of a commercial and an experimental dairy plant, they found the microflora included *Lactobacillus, Streptococcus, Lactococcus, Staphyloccous* and

Micrococcus species. Therefore, the biofilm formed from the 2% M.F pasteurized milk was most likely a multi-species consortium containing various bacterial species.

5.5 The ability of Phytochemicals to Remove Preformed Biofilms In vitro

Biofilms from 2% M.F pasteurized milk microbiota with the addition of *L. monocytogenes* at 100 CFU/ml were allowed to form mature biomass (74 hours) in a 96 well plate based on the biofilm growth kinetics previously determined for non-spiked samples. This created a worst-case scenario for examining the persistence of *L. monocytogenes* as harbourage of the pathogen within mixed-species biofilms greatly enhances its resistant relative to its pure culture biofilms (Gandhi and Chikindas, 2007). As determined by the crystal violet assay the phytochemicals that gave the greatest reductions in biofilm biomass were tea tree EO, thymol, carvacrol, coriander EO and eugenol. No significant difference was observed between the exposure period or the concentrations of the phytochemicals in regard to to their ability to remove the preformed biofilm. Sandasi et al (2009) also showed that culinary herbs (oregano and thyme) and their derivatives (carvacrol, thymol, and eugenol) were able to remove significantly greater amounts of biomass from preformed biofilms than other essential oils.

5.6 Enumeration of *L. monocytogenes* in Dispensing Pumps

Investigating the ability of *L. monocytogenes* strain Lm-F-92 to attach to internal surfaces and replicate within a milk dispensing system demonstrated that the pathogen cell numbers significantly increased over a nine-day period, despite the system being kept at 4°C. Enumeration of viable *L. monocytogenes* within the pumps and tubing over the nine days showed a continuous increase in cell numbers. Although the pathogen could not be detected on day 0 of the experiment, on day 3 the counts were 5.25×10^1 CFU/ml, which subsequently increased to 2.45×10^3 CFU/ml and 6.14×10^4 CFU/ml on days 6 and 9, respectively. As *L. monocytogenes* have the

ability to attach to polystyrene, as well as various other surfaces, many of which are materials found inside the surfaces of the pump (Shi and Zhu, 2009), it was expected to see an increase in the number of *L. monocytogenes*. These values observed indicate a need to achieve 100% removal of biomass within the pump throughout the cleaning procedure in order to ensure the safety of the product as the infectious dose of *L. monocytogenes* is 1 CFU/ml for high-risk food such as milk (Government of Canada, 2011).

5.7 The Ability of Phytochemicals to Removed Preformed Biofilms in Dispensing Pumps

To establish a cleaning procedure for the commercial milk dispensing units, the pumps and tubing of the milk dispensing units were filled with 2% M.F pasteurized milk and left for 74 hours at 4°C for a mature biofilm to form. The milk was later replaced by the phytochemicals previously determined to remove biomass in the 96 well plates following an exposure time of 1 hour and at a high concentration. Unfortunately, none of the phytochemicals were able to significantly remove the preformed biofilms in the pumps and tubing. Surprising was the fact that tea tree EO and coriander EO were found to increase biomass of the preformed biofilms by 13% and 24%, respectively. This could be due to the essential oils encouraging the attachment of other bacterial cells or particles found in the milk. Whereas thymol reduced biomass by 17.0%, carvacrol reduced by 8.5% and eugenol reduced by 4.8%. These results are in agreement with other studies such as those of Sandasi et al (2009) and Walmiki and Rai (2017), which showed the ability of EOs to remove preformed biofilms to be limited; with better results obtained when the EOs were used as a pre-treatment to discourage cells from attaching to surfaces.

Possible speculative explanations for the large differences in biofilm removal between microtiter plates and the rotary pumps could include the nature of the substrate, which in turn could influence the biofilm characteristics like thickness, density and type of matrix produced. In

addition, it should be noted that while the microtiter plate experiments could be run with milk from the same carton (as they required small volumes of milk), the pump experiments used milk from different cartons, possibly having a slightly different microbiota. Future research could focus on finding the conditions under which biofilm removal from the pumps would resemble what was observed in the microtiter plates.

5.8 The ability of Phytochemicals to Remove Preformed Biofilms With the Addition of Mechanical Action

Often mechanical force is utilized to remove preform biofilms. Therefore, since carvacrol and thymol had some effect removing the biofilm when introduced statically, additional experiments were carried out where these phytochemicals were subjected to the preform biofilms in the pumps and tubing while being continuously circulated for 15 minutes, then resting for 15 minutes followed by a final 15-minute circulation. Neither carvacrol nor thymol was able to remove significant amounts of the preformed biomass; however, the circulation of the phytochemicals result in improved reductions where thymol was able to remove 32% of the biomass and carvacrol 23% of the biomass. Therefore, continued investigations are required regarding the efficacy of phytochemicals as potential cleaning agents for commercial milk dispensing units to ensure a high level of food safety.

5.9 Limitations

This study was incumbered by a number of limitations. When screening the phytochemicals against the two *Listeria* species, the biggest limitation was time. Additional time would have allowed a greater number of compounds to be screened individually or in combinations, to achieve the desired outcome of greater inhibition of *L. innocua* over *L. monocytogenes* to explore the implementation of phytochemicals in the method MFHBP-30. A

lack of literature around selectively choosing *L. monocytogenes* over *L. innocua* provided the opportunity to verify the range of dosage as well as *L. monocytogenes* susceptibility to the various phytochemicals tested, however; additional time was taken to identify the MIC for compounds used.

Limitations that arose throughout the experiments involving the milk dispensing units, were largely related to consistency. Although each experiment was conducted three times, much variation occurred with the results in each trial, which resulted in large standard deviations. The variations were thought to be a result of the pumps losing uniformity as wear on the pumps occurred as there were experimental parts being utilized throughout the system. As was evident with the differences in results between the *in vitro* and *in situ* experiments utilizing the plant secondary metabolites to remove biofilm without mechanical action, the environment within the pumps was difficult to replicate in vitro with the polystyrene 96-well microtiter plates. A remedy for these limitations would have a larger number of pumps and milk dispensing units to have higher throughput to achieve more accurate results.

CHAPTER 6 CONCLUSION

6.1 Project Summary

Firstly, the ability of phytochemicals to differentially inhibit *L. monocytogenes* and *L. innocua* was investigated. Overall, fourteen of the individual phytochemicals and five of the combinations tested were found to significantly differentiate between the two *Listeria* species. Most favored the growth of *L. innocua* over *L. monocytogenes*; however, as a group, the efflux pump inhibitors resulting in significant differences were found to favor the growth of *L. monocytogenes* over *L. innocua* in the UVM1 broth. These compounds were ferruginol, silibinin, NMP, and omeprazole.

Theaflavin, cinnamon bark EO and ferruginol, and cinnamon bark EO and NMP were then screened against twelve strains of *L. monocytogenes* and twelve strains of *L. innocua.* With the combination, cinnamon bark EO and ferruginol, being able to cause significant differentiation amongst the twenty-four strains. Theaflavin (≥90% Purity) was noteworthy as it was able to inhibit all *Listeria* strains except one *L. innocua* strain (L. inno (11)). Unfortunately, none of the phytochemicals were able to select for *L. monocytogenes* while completely inhibiting *L. innocua.*

Secondly, the ability of phytochemicals to remove preformed biofilms in the pump and tubing of milk dispensing units was investigated. To start, the growth kinetics of the biofilm formed from the 2% M.F pasteurized milk microbiota was determined. It was found that mature biofilm occurred at 74 hours when 2% M.F pasteurized milk is incubated at 4°C in polystyrene 96 well microtiter plates. With the addition of *L. monocytogenes*, mature biofilms were allowed to form in the wells of 96 well plates. These biofilms were then subjected to phytochemicals for 1-hour or 5-hour exposure at a high and low concentration. The % reduction in biomass was then determined, revealing that tea tree EO, thymol, carvacrol, coriander EO and eugenol were able to reduce the biomass within a range of 89% to 97%. After determining that *L. monocytogenes*

could harbor and replicate within the pump and tubes of the milk dispensing units over 9 days 4°C, the phytochemcials were tested to determine their effectiveness at removing the mature biofilm within the pumps and tubing. None of the compounds were able to remove significant amounts of biomass with thymol reducing the most at 17%, followed by carvacrol at 8.5%, then eugenol at 4.8%. Mechanical action was then employed in combination with thymol and carvacrol treatment to assess whether the removal of preformed biofilms could be improved. The mechanical action helped remove more biomass than static exposure alone; however, thymol was only able to reduce biomass by 32% and carvacrol reduced biomass by 23%.

This study successfully proved that phytochemicals have an inhibitory effect on *L. monocytogenes* as well as anti-biofilm characteristics. Further research is needed to identify a compound that successfully selects *L. monocytogenes* over *L. innocua*; however, the compounds already screened showed that efflux pump inhibitors are promising choices. To ensure food safety within the milk dispensing units, further exploration of anti-biofilm compounds is needed to obtain 100% removal of biomass within the pumps and tubing.

This is an accordance with other studies that find MIC values of phytochemicals to be in a range of 100 µg/ml to 1000 µg/ml (Tegos et al, 2002). Both of these compounds are expelled by efflux pumps in *L. monocytogenes* causing them to be resistant to the dye and antibiotic, however, with the addition of the efflux pump inhibitor in the UVM broth, *L. monocytogenes* was then susceptible to these compounds. Again, these values are lower as EOs are a combination of active phytochemicals that create a synergistic effect against *L. monocytogenes*.

6.2 Future Directions

1. Screen additional efflux pump inhibitors against *L. monocytogenes* and *L. innocua* as several tested in the current study allowed for favored growth for *L. monocytogenes* over *L. innocua.*

2. Investigate the effect of phytochemical purity on the antimicrobial activities.

3. Further analysis of the *L. innocua* strain, L.inno (11), should occur to determine how it was able to overcome the inhibitory effect of theaflavin (Sigma-Aldrich).

4. Utilize combinations of phytochemicals to remove preformed biofilms to achieve greater removal of biomass.

5. Investigate the effectiveness of efflux pump inhibitors to remove preformed biofilms within the milk dispensing units.

6. Explore the effects of the phytochemicals when *L. monocytogenes* and *L. innocua* are mixed together *in vitro*.

7. Identify the mechanism of action for the differential selection effect between *L. monocytogenes* and *L. innocua* in the UVM1 broth through whole-genome sequencing.

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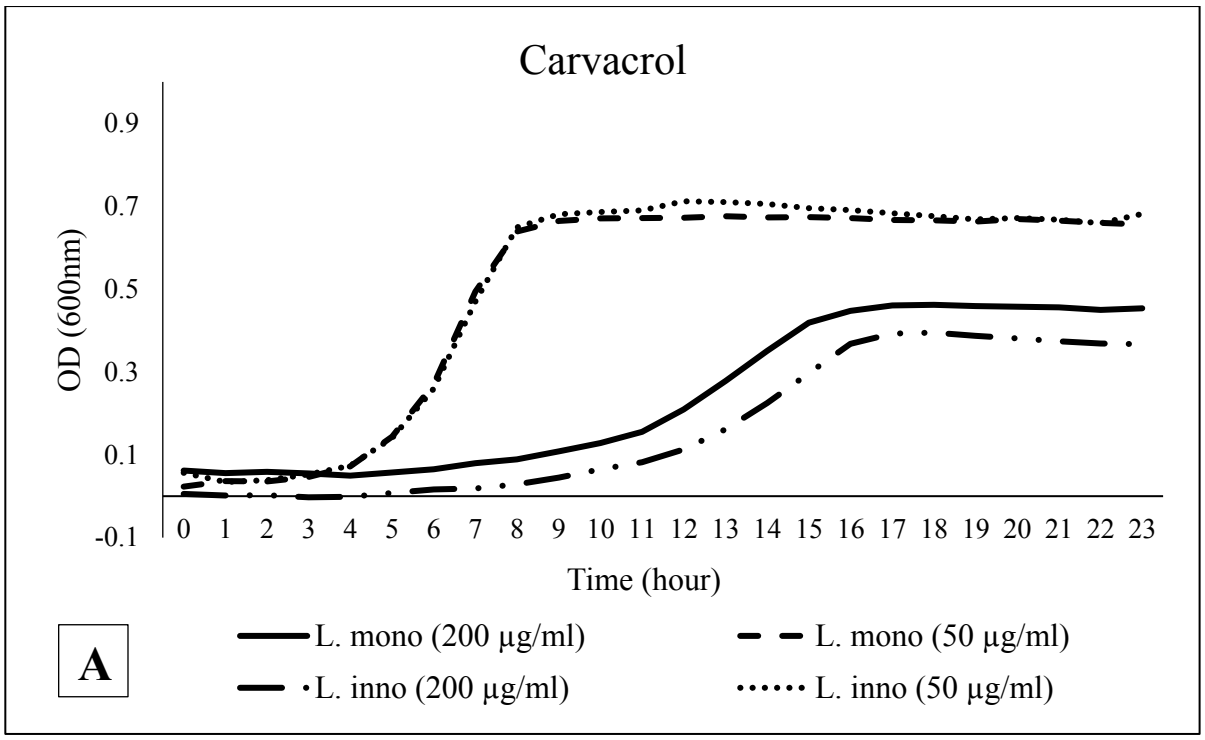
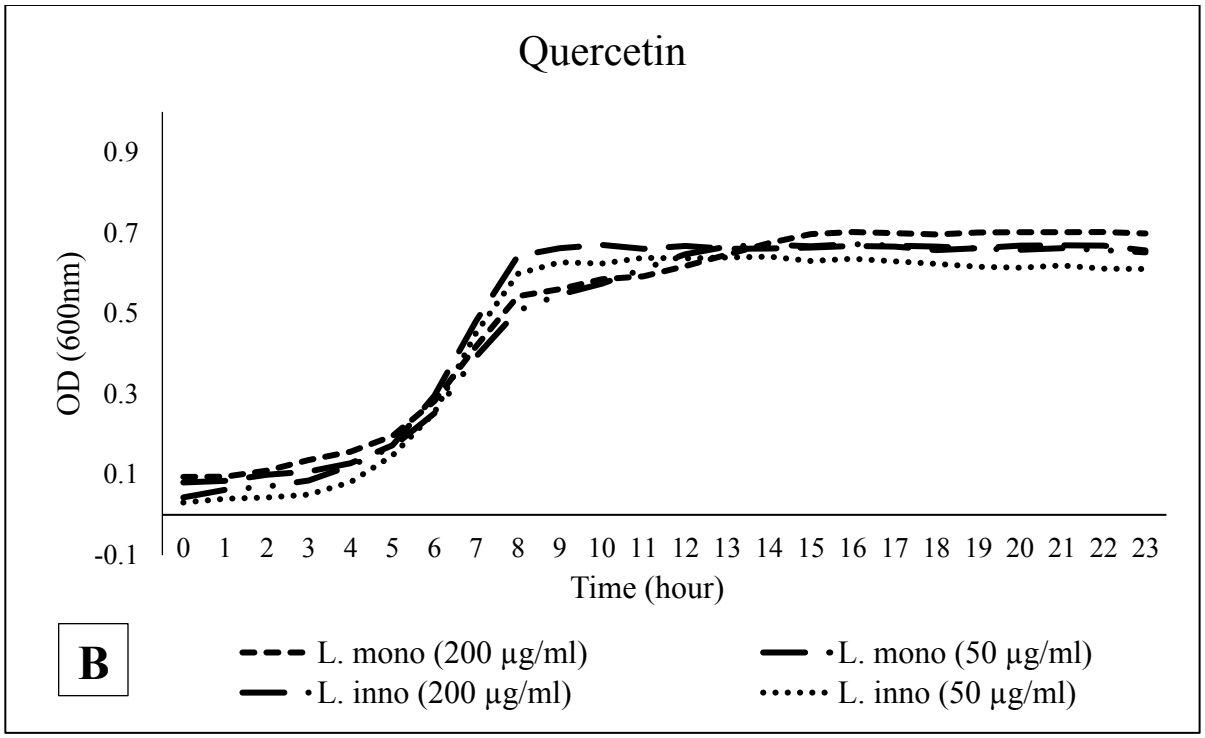
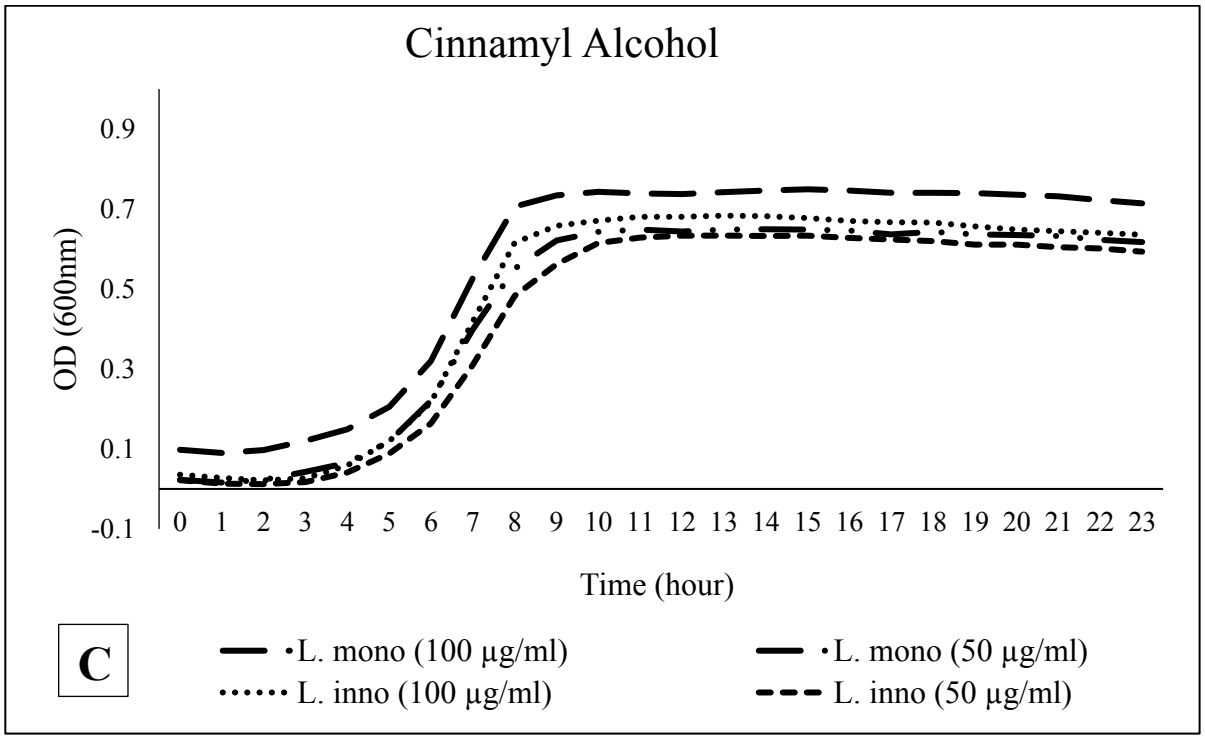
APPENDIX

The growth curves and bar graphs of the phytochemcials that were determined to not have a significant differential effect between *L. monocytogenes* and *L. innocua* are shown below.

Initial Screening of Phytochemicals with General Antimicrobial Properties against *L. monocytogenes* and *L. innocua*




Quercetin
0.9
0.7
0.5
0.3
0.1
-0.1
OD (600nm)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (hour)
B
L. mono (200 µg/ml)
L. mono (50 µg/ml)
L. inno (200 µg/ml)
L. inno (50 µg/ml)


Cinnamyl Alcohol
0.9
0.7
0.5
0.3
0.1
-0.1
OD (600nm)
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (hour)
C
L. mono (100 µg/ml)
L. mono (50 µg/ml)
L. inno (100 µg/ml)
L. inno (50 µg/ml)

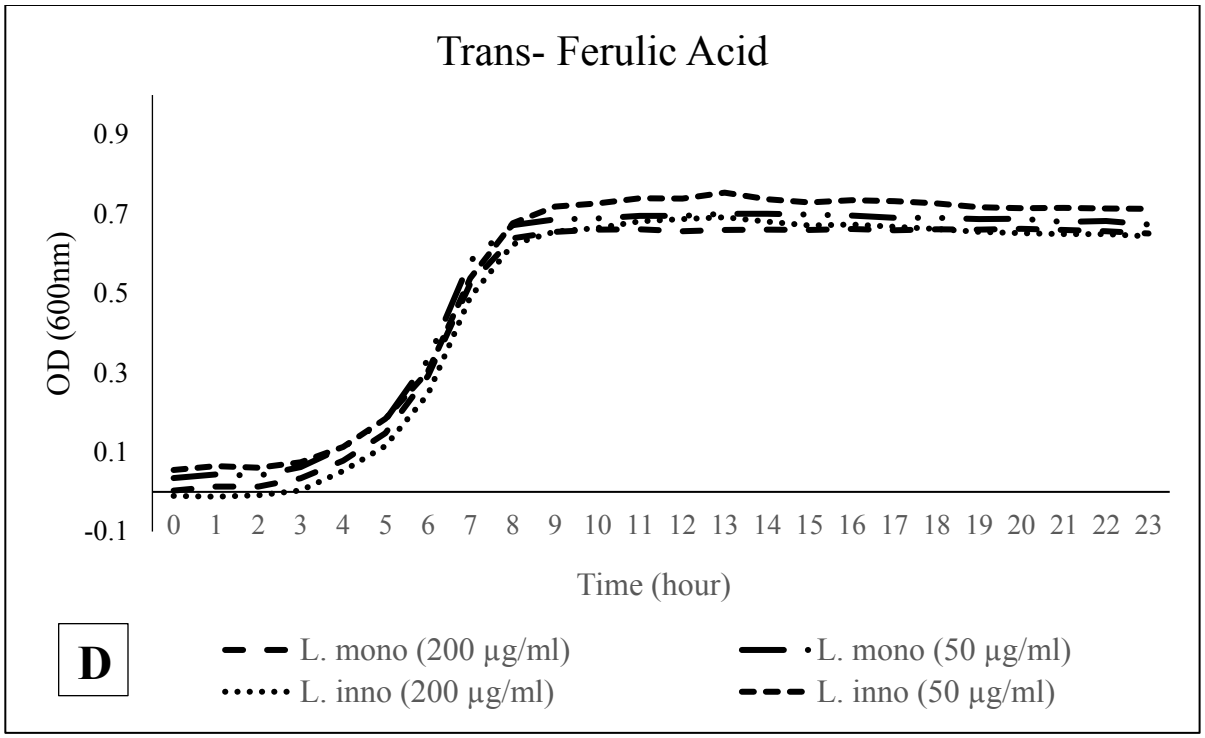
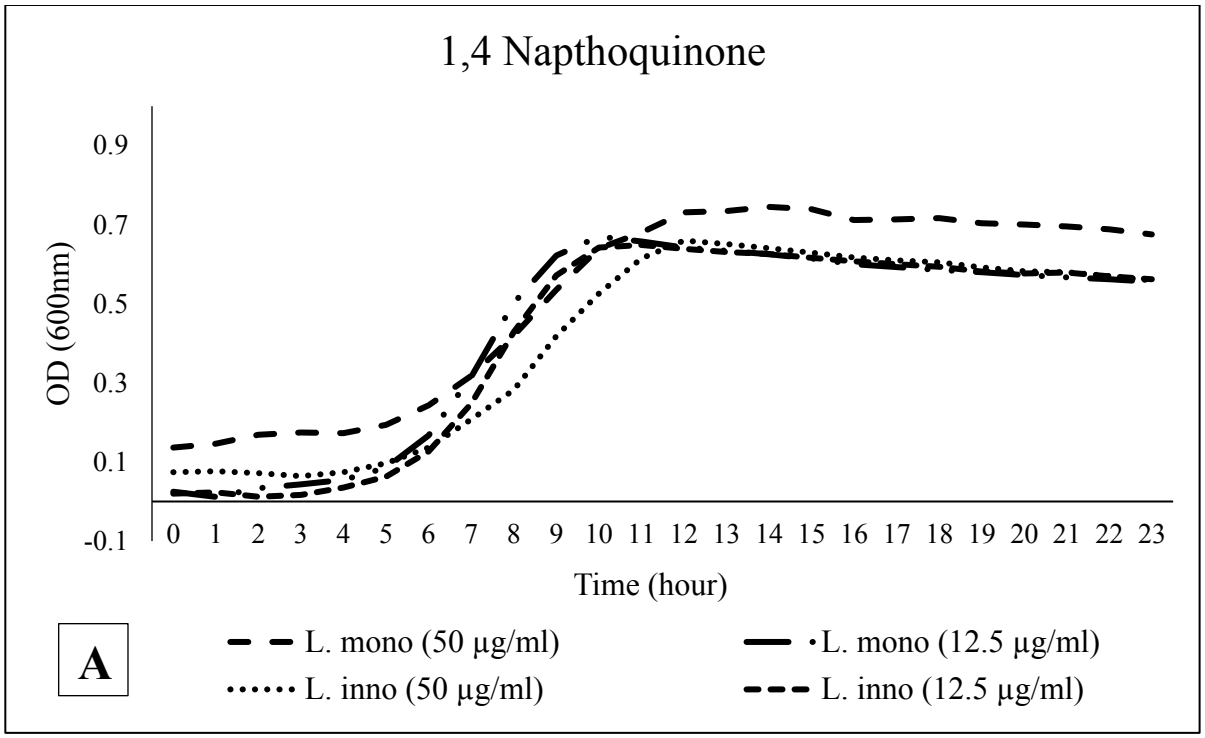
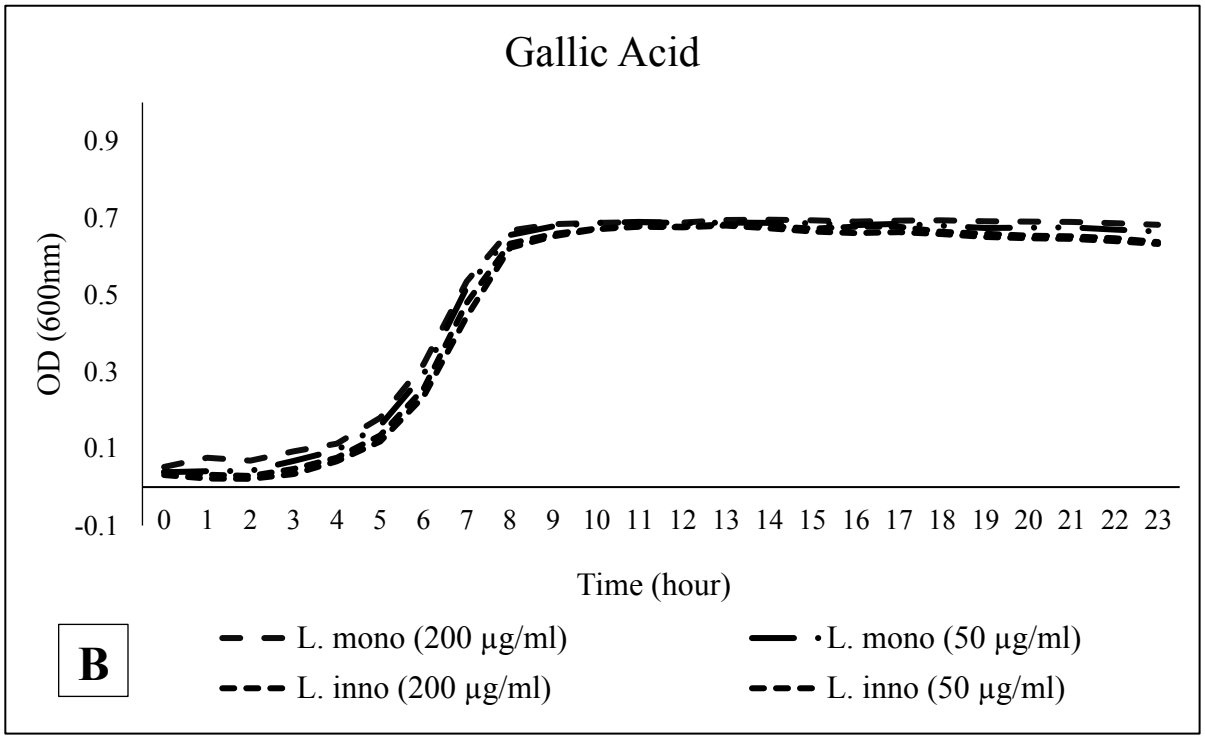
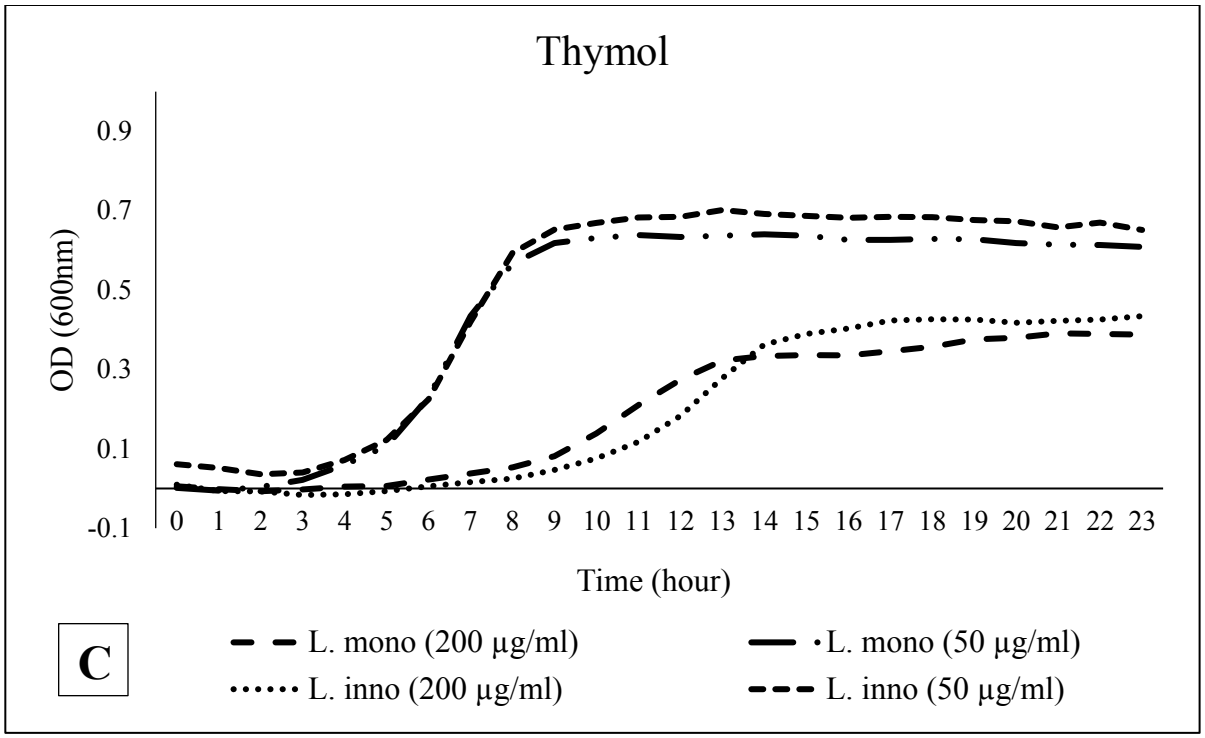


Figure 17: Initial screening (time vs OD_{600nm}) of (A) Carvacrol, (B) Quercetin, (C), Cinnamyl Alcohol and (D) Trans-Ferulic Acid against *L. monocytogenes* FSL-J1-031 and *L. innocua* 2007-663. These compounds were found not to have any significant difference on the two strains of *Listeria*. (n=9).

Initial Screening of Additional General Antimicrobial Phytochemicals Against *L. monocytogenes* and *L. innouca*


1,4 Napthoquinone
OD (600nm)
0.9
0.7
0.5
0.3
0.1
-0.1
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (hour)
A
L. mono (50 µg/ml)
L. mono (12.5 µg/ml)
L. inno (50 µg/ml)
L. inno (12.5 µg/ml)


Gallic Acid
OD (600nm)
0.9
0.7
0.5
0.3
0.1
-0.1
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (hour)
B
L. mono (200 µg/ml)
L. mono (50 µg/ml)
L. inno (200 µg/ml)
L. inno (50 µg/ml)



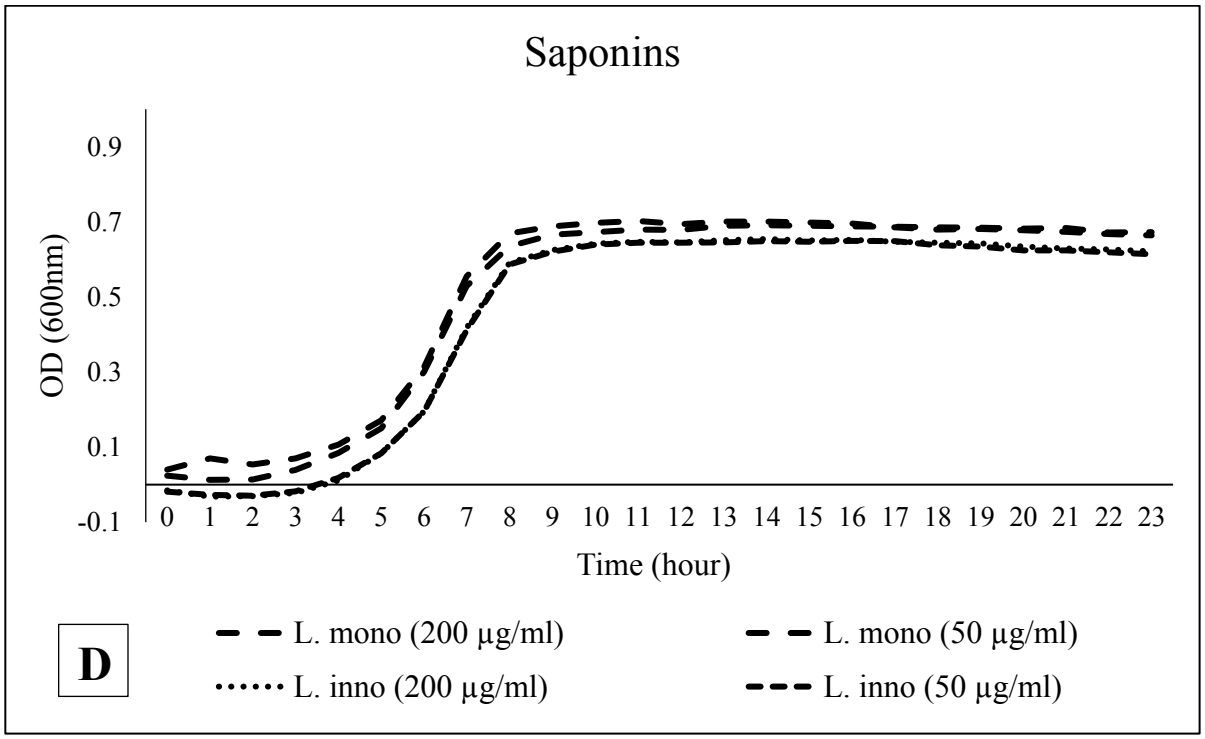
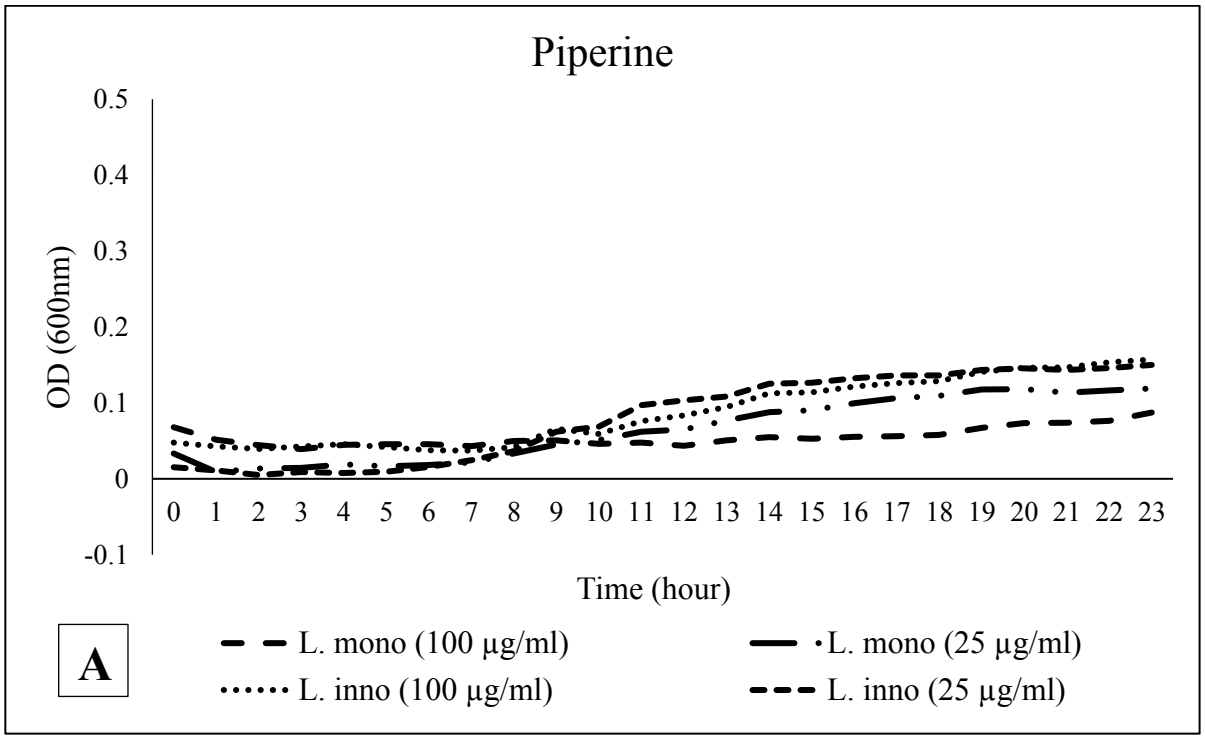
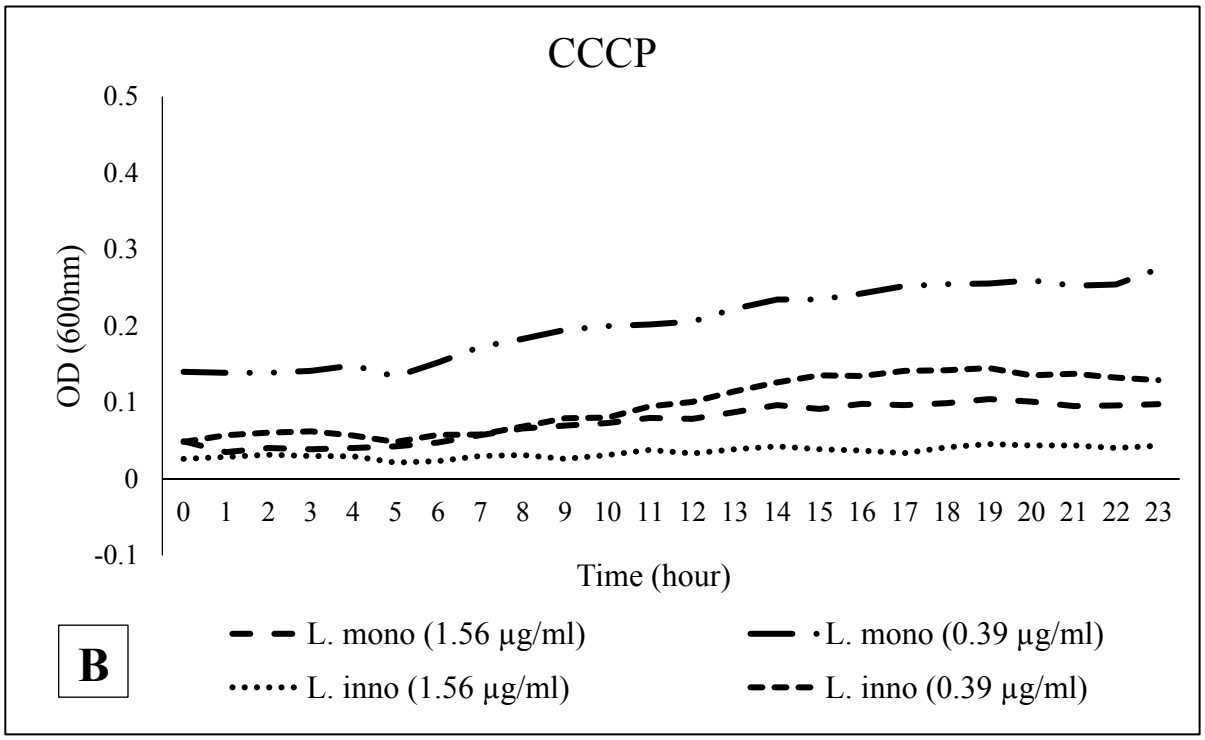


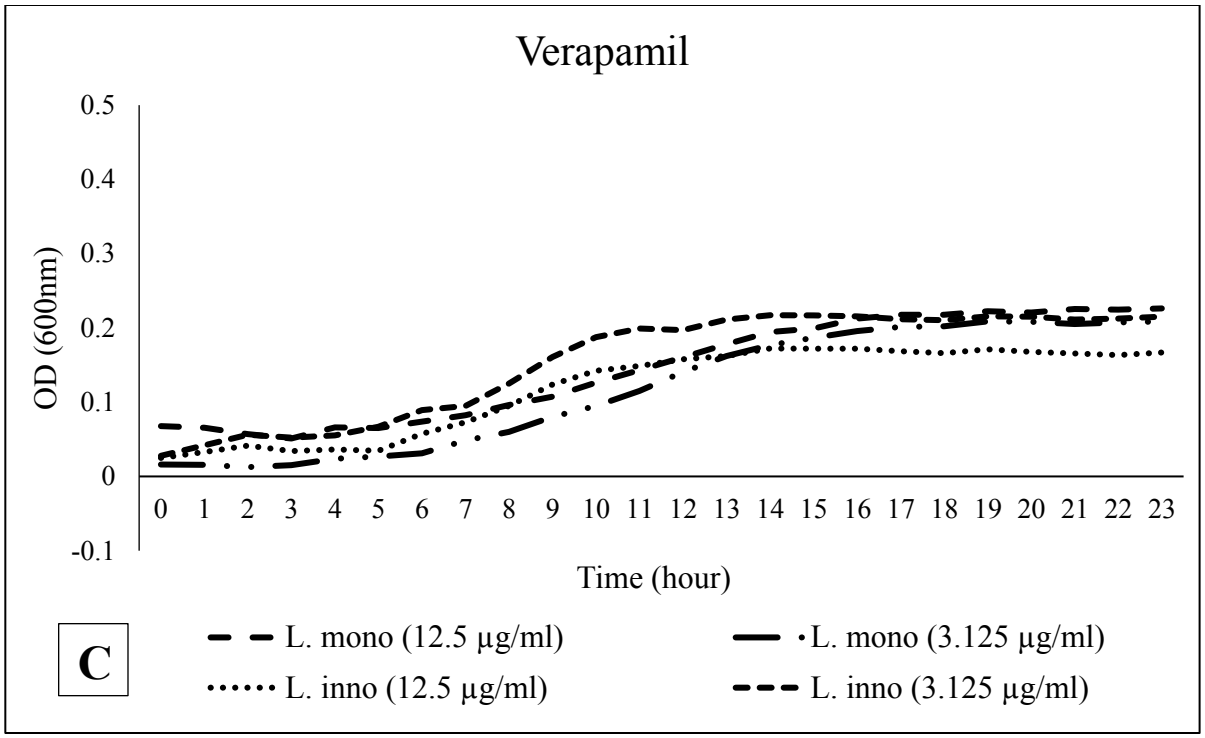
Figure 18: Initial screening (time vs OD_{600nm}) of (A) 1,4 Napthoquinone, (B) Gallic Acid, (C) Thymol and (D) Saponins against *L. monocytogenes* FSL-J1-031 and *L. innocua* 2007-663.

These compounds were found to not significantly differentiate between the two strains of *Listeria.* (n=9).

Initial Screening of Efflux Pump Inhibitors against *L. monocytogenes* and *L. innouca*







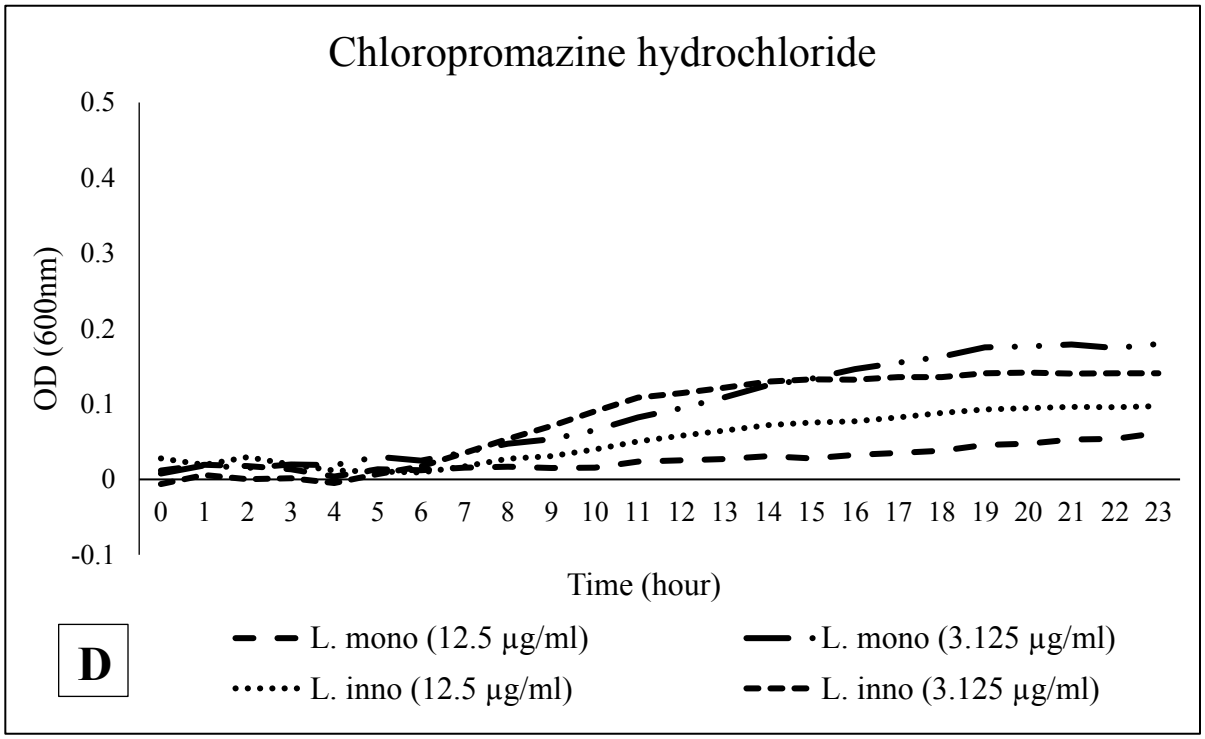


Figure 19: Initial screening (time vs OD_{600nm}) of efflux pump inhibitors (A) Piperine, (B) CCCP, (C) Verapamil and (D) Chloropromazine hydrochloride against *L. monocytogenes* FSL-J1-031

and *L. innocua* 2007-663 at a high and low concentration. These compounds did not show any significant differential effect against the two *Listeria* strains. (n=9).

Initial Screening of Tea Tree EO against *L. monocytogenes* and *L. innouca*

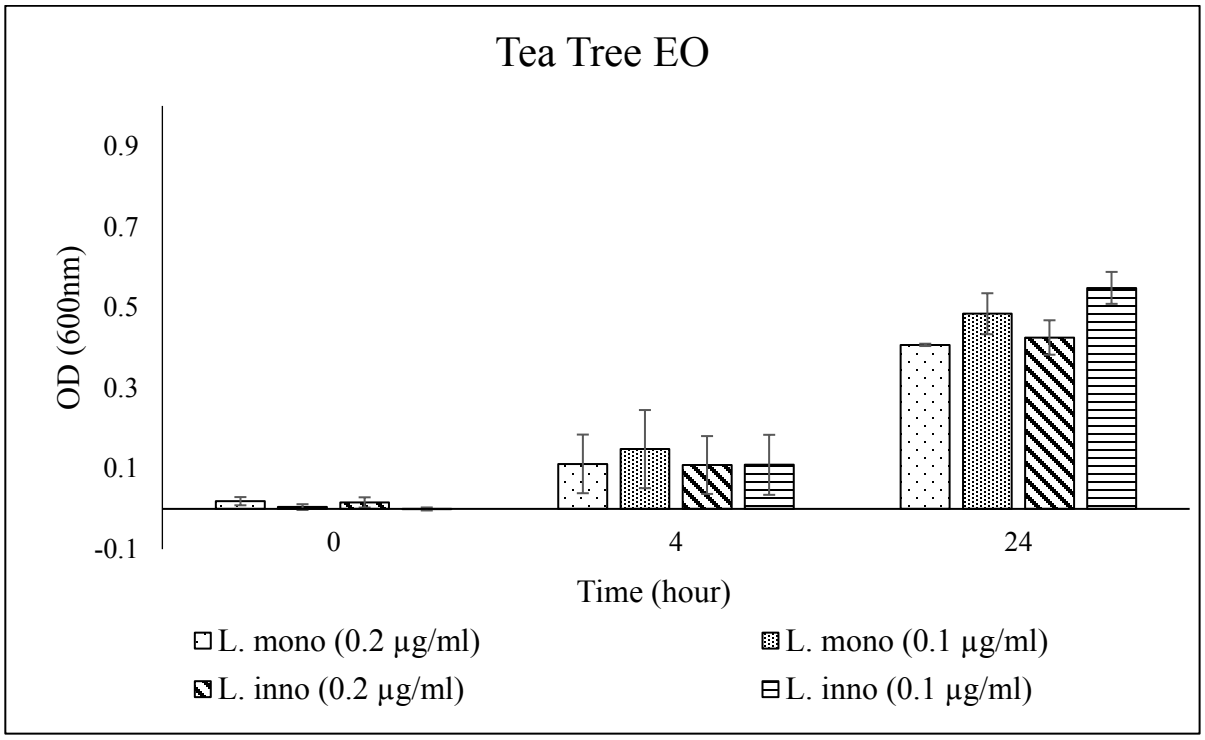
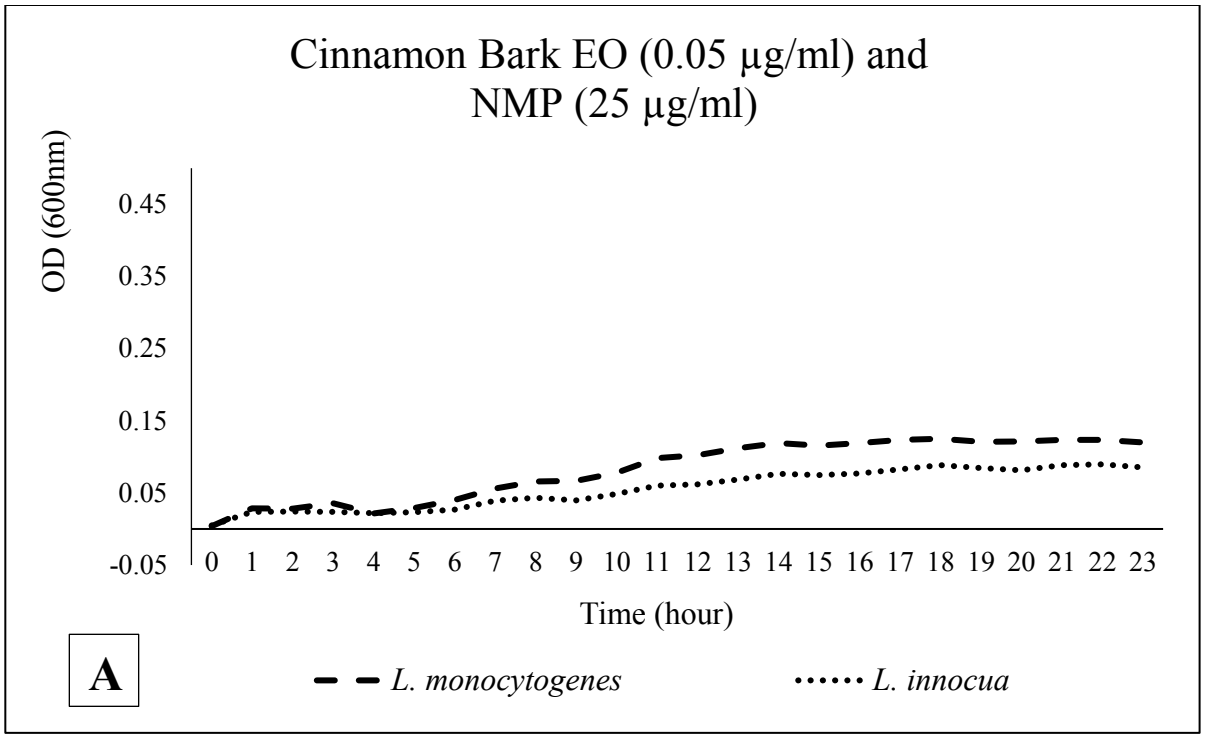
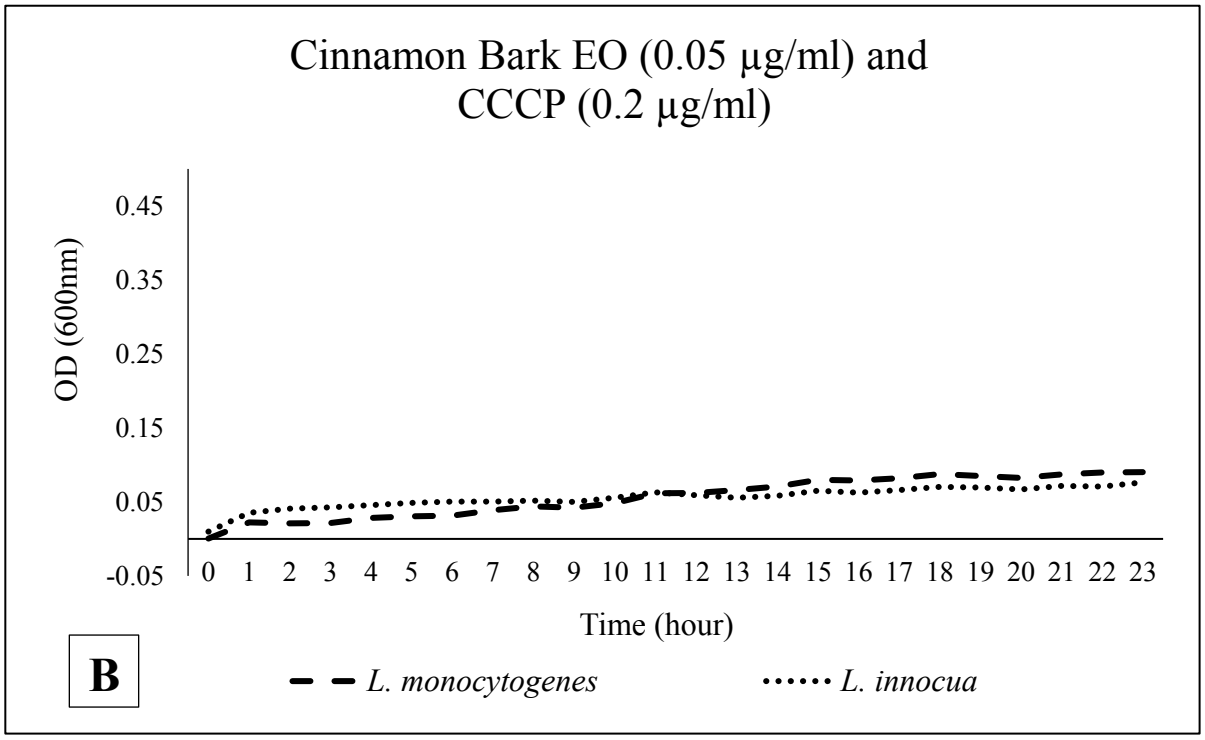
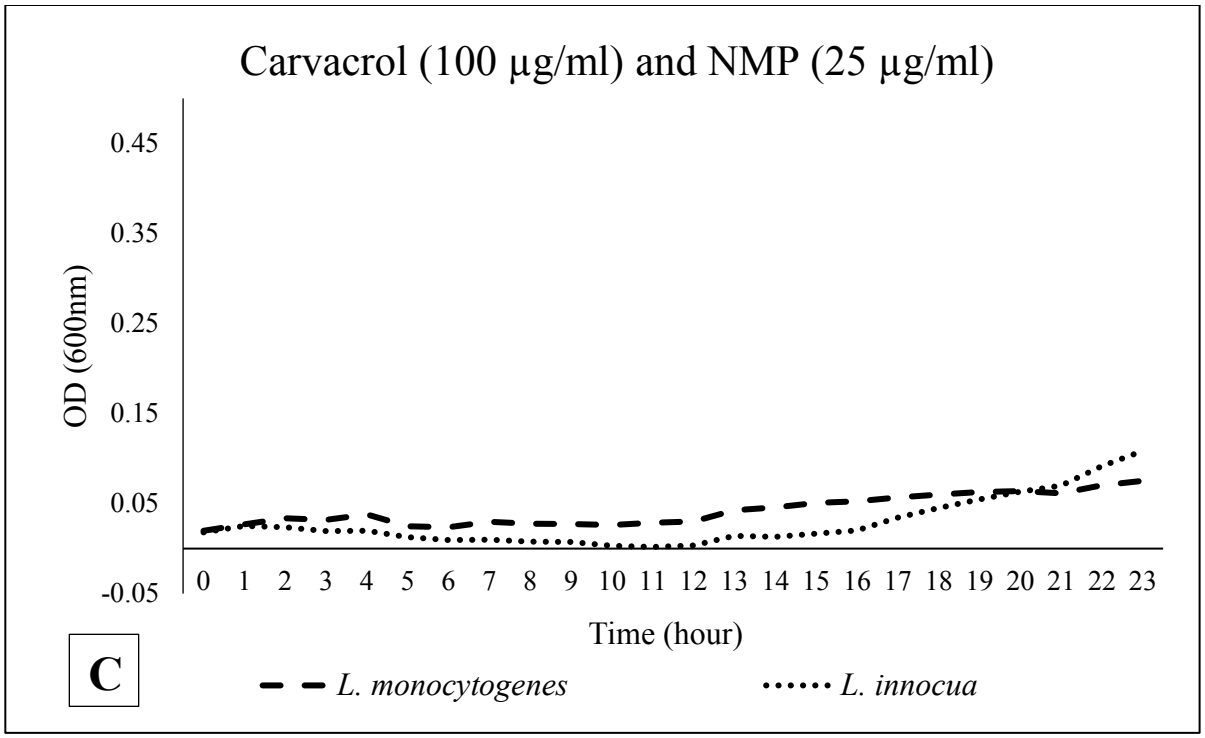


Figure 20: Initial screening (time vs OD_{600nm}) of tea tree essential oil against *L. monocytogenes* FSL-J1-031 and *L. innocua* 2007-663. Tea tree EO did not show any significant differential effect between the two *Listeria* species. Error bars represent standard deviation. (n=9).

Initial Screening of Combinations of Phytochemicals Against *L. monocytogenes* and *L. innouca*


Cinnamon Bark EO (0.05 µg/ml) and
NMP (25 µg/ml)
OD (600nm)
0.45
0.35
0.25
0.15
0.05
-0.05
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (hour)
A
L. monocytogenes
L. innocua


Cinnamon Bark EO (0.05 µg/ml) and
CCCP (0.2 µg/ml)
OD (600nm)
0.45
0.35
0.25
0.15
0.05
-0.05
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23
Time (hour)
B
L. monocytogenes
L. innocua



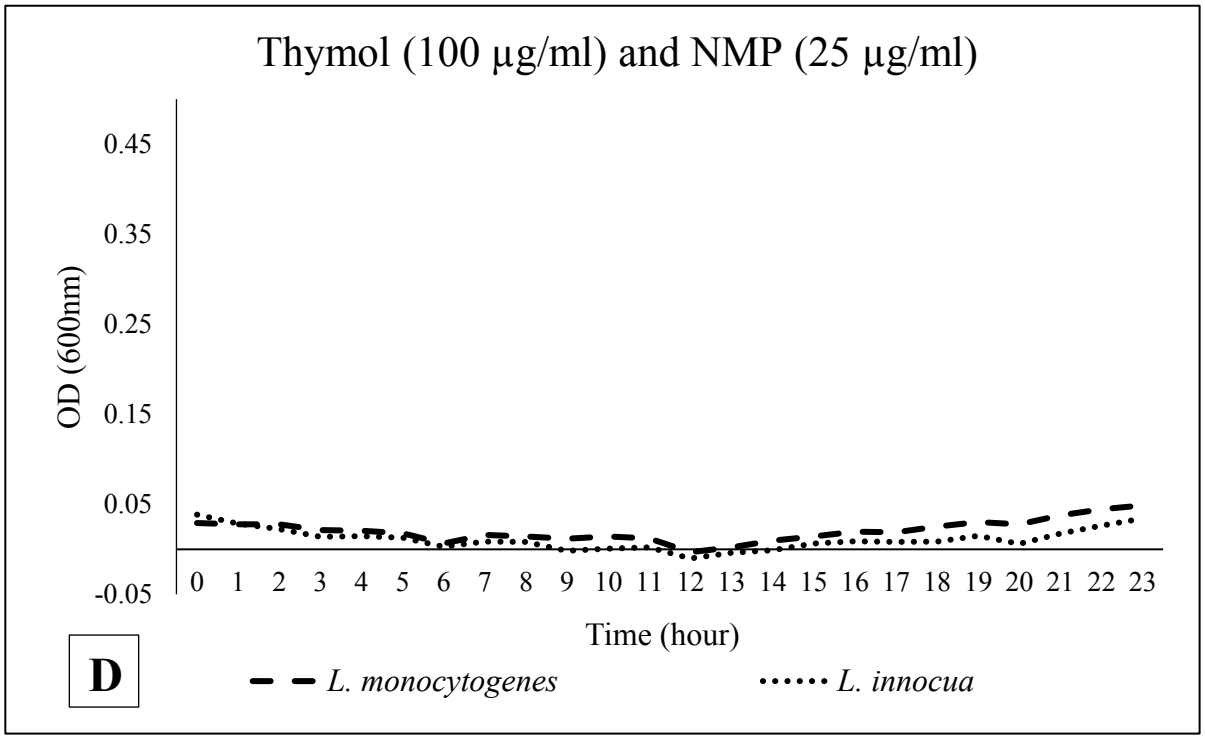


Figure 21: Initial screening (time vs OD_{600nm}) of the combinations of (A) Cinnamon Bark EO and NMP, (B) Cinnamon Bark EO and CCCP, (C) Carvacrol and NMP and (D) Thymol and NMP

against *L. monocytogenes* FSL-J1-031 and *L. innocua* 2007-663. These combinations were found not have a significant differential effect between the two *Listeria* strains. (n=9).

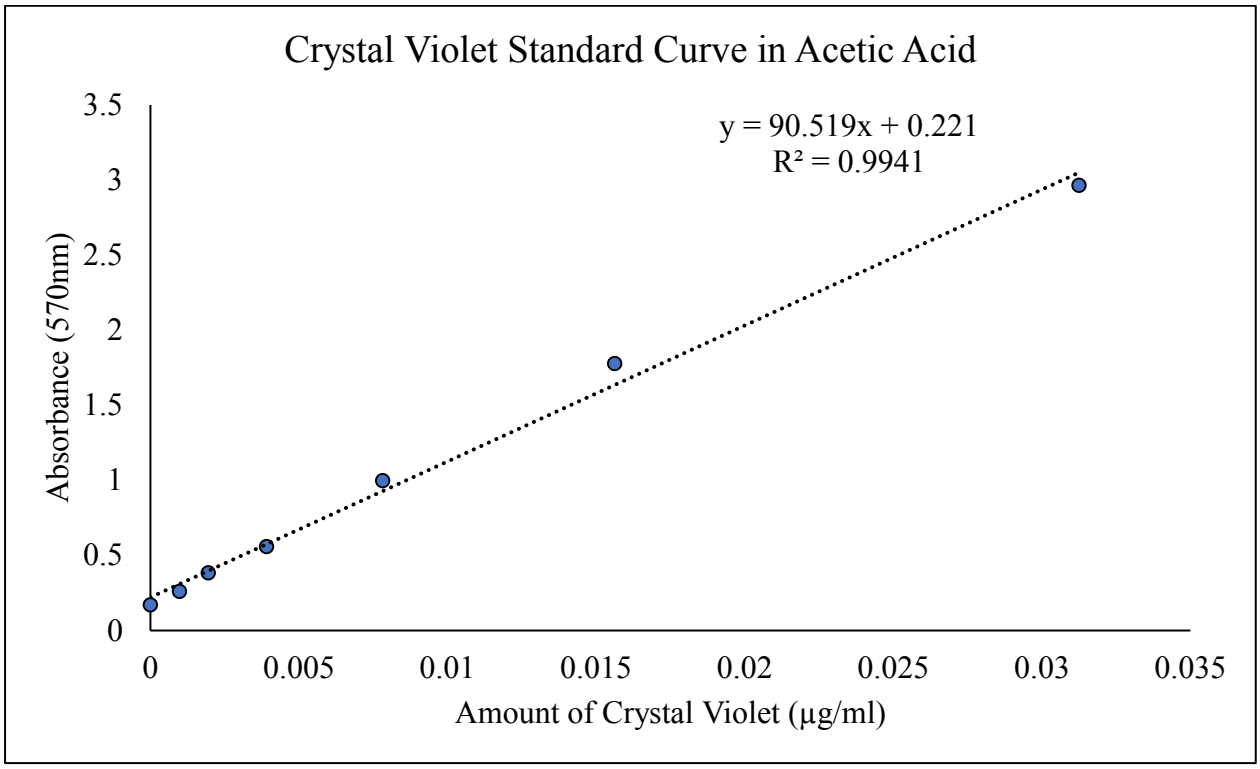


Figure 22: Standard curve used to determine the concentration of crystal violet in the 96-well microtiter plates.

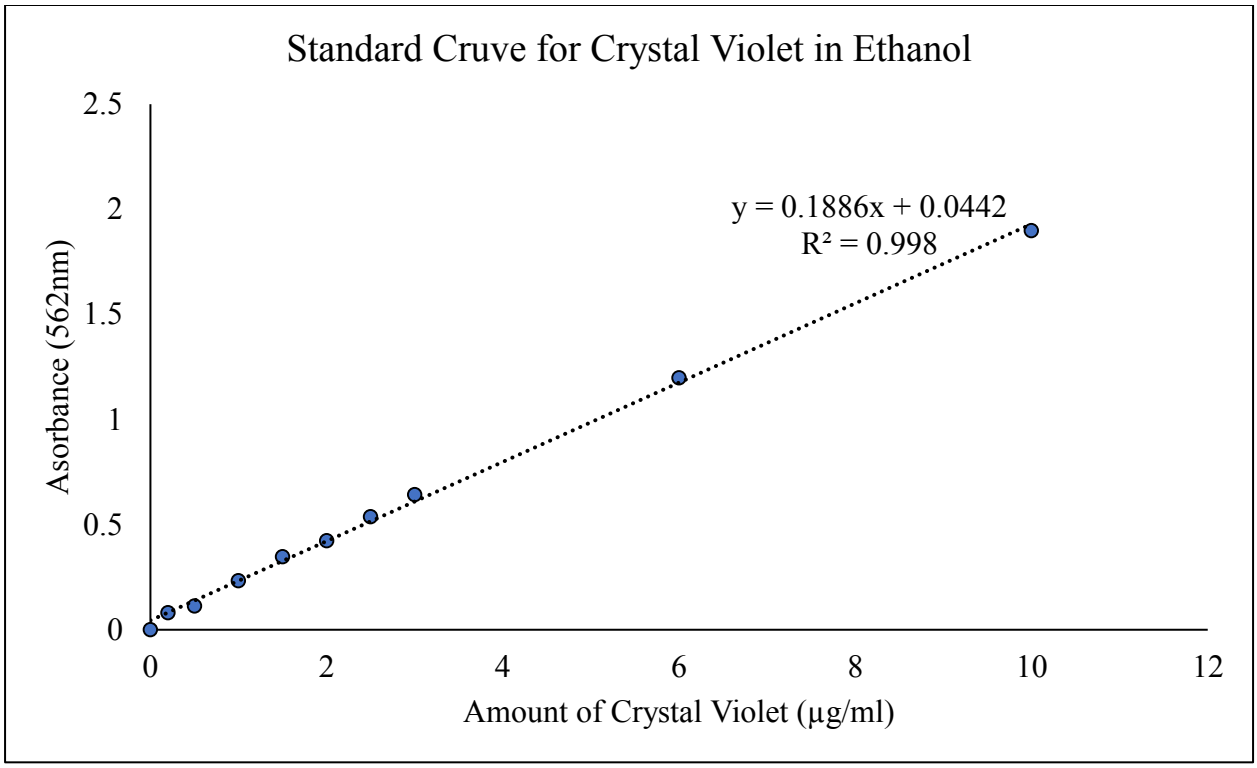


Figure 23: Standard Curve used to determine concentration of crystal violet in milk dispensing pumps.