Application of thyme oil or cinnamaldehyde for the inactivation of enteric pathogens in marinated raw chicken and apple juice

by

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

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TABLE OF CONTENTS

ABSTRACT

Non-thermal antimicrobial interventions have recently gained much attention as there is a change in consumer patterns who now demand foods manufactured by minimal processing and are free of synthetic chemical additives. This thesis discusses the application of essential oils (thyme oil) and their components (cinnamaldehyde) to control of foodborne pathogens such as *Salmonella* and *Listeria* that are responsible for numerous deaths, hospitalizations and food product recalls. Poultry meat is one of the common vehicles of transmission of Non-typhoidal *Salmonella* and thus we proposed the application of thyme oil to assess the survivability of *Salmonella enterica* artificially inoculated in chicken breast meat, marinated in a lemon-based marinade containing varying levels of thyme oil. Cells in the long-term phase have been found to be more tolerant to heat, cold plasma, high pressure processing or UV-radiation. The second objective of the thesis addresses the tolerance *Listeria* and *Salmonella* in the long-term survival phase exhibit when exposed to cinnamaldehyde in saline and apple juice compared to cells in the stationary phase. The results demonstrate that essential oils have are potent antimicrobials and thus can be used to enhance safety and extend shelf life of food.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

The bacteria responsible for food spoilage or transmission of foodborne illness remain a significant threat to the food industry and the to the public, globally. Latest epidemiologic reports by the Center for Disease Control (CDC) indicate that 1 in 6 Americans suffer from a foodborne ailment annually, 128,000 people are hospitalized, and an estimated 3,000 people succumb to death from various illnesses contracted from the consumption of contaminated food. The number of people who get ill from foodborne microbial infections are as follows: norovirus (2.2 million), *Salmonella* (3.6 million), *Campylobacter spp.* (1.9 million) and *Clostridium perfringens* (342,000). The annual number of cases of foodborne infection linked to *L. monocytogenes* and *Escherichia coli* OH157:H7 are generally lower that of other foodborne pathogens such as *Salmonell*a, *Campylobacter* and enteric viruses; however, those two pathogens still contribute significantly to the food safety problem in the modern world. For example, *Listeria monocytogenes* has the highest mortality rates especially amongst immunocompromised individuals and *E. coli* O157:H7 can cause life-threatening diseases such as hemorrhagic colitis, hemolytic uremic syndrome and thrombotic thrombocytopenic purpura.

To control spoilage and pathogenic bacteria, the food industry has always employed interventions such as heat pasteurization, canning, acidification, drying, salting, smoking, and synthetic antimicrobial agents to make the food safe for human consumption. However, consumers are increasingly demanding for more natural antimicrobial interventions prompting the shift from traditional methods of food preservation. Consumers associate heat treatments with reduced or degraded nutrients while they are skeptical of the health consequences associated with prolonged exposure to chemical food preservatives as some studies have reported them to be carcinogenic or

lead to teratogenicity or acute toxicity in the long run (Faleiro, 2011; Calo et al., 2015). The development and adaptation of alternative and natural substitutes to preserve food is on the rise. Essential oils and their components such as thyme oil and cinnamaldehyde have shown tremendous potential as alternatives to chemical food preservatives because they are plant extracts with potent antimicrobial components. The main challenges impeding their application in food is that they confer strong odors to foods altering them organoleptically. Also, they are expensive to extract compared to the synthesis of chemical preservatives such as sorbates and benzoates.

Like any living organism, survival and continuation of species is important and bacteria are not any different. Conventionally bacteria have been known to have four stages in their life cycle namely, lag, exponential, stationary and the death phase. However, not all bacteria die at the death phase leading to the transition into a fifth phase to ensure the survival of the next generation of daughter cells (Kolter et al., 1993). Because of constant exposure to stress, the cells that survive at the death phase transition into a fifth stage named the long-term survival phase. The transition from stationary to long-term survival (LTS) phase is a survival mechanism associated with exposure of foodborne pathogens to constant stress like that afflicted on them by antimicrobial practices utilized by the food industry. Such stresses include heat, sanitizers or artificially synthesized chemical preservatives and environmental stresses such as desiccation, nutrient depletion or accumulation of metabolic waste. The transition to the LTS phase has also been shown to equip non-spore forming bacteria with physiological and metabolic 'tools' that increases the cells chances of survival by increasing their tolerance to antimicrobial interventions that will be discussed later in this thesis.

Studies have shown that LTS phase cells have increased tolerance to antimicrobial interventions compared to stationary phase cells. Wen et al. (2009) demonstrated that LTS cells of

strains of *L. monocytogenes* had increased tolerance to antimicrobial interventions such as heat and high pressure while Wang et al. (2018) showed that LTS phase cells of *S.* Typhimurium were more resistant to UV radiation compared to stationary phase cells of that same pathogen. The increased tolerance of LTS phase cells poses a new threat to the food industry and advancement of non-thermal technologies in preserving food because most industrial processes designed to kill microorganisms in food are designed using microbial challenge studies utilizing stationary phase cells that are less robust (Wang et al., 2018). In this respect, an important question is whether LTS phase cells should be adopted as the new standard to validate new antimicrobial processes. As we continue with the practice of using stationary phase cells, the number of food recalls associated with the occurrence of foodborne pathogens keeps on rising despite the advancement in modern-day technology.

Thesis Organization

This thesis is divided into five chapters with chapter 1 consisting of the general introduction, giving insights to the work that is explained in the subsequent chapters. Chapter 2 consists of literature review that provides deeper explanations on the research conducted in chapters 3 and 4. Chapters 3 and 4 consist of research manuscripts that will be submitted to the journal of Poultry Science and the Foodborne Pathogens and Disease journal, respectively. The references, tables and figures are found at the end of each chapter. Chapter 5 provides the summarizes and the general conclusions to the findings of the two studies in this thesis.

CHAPTER 2. LITERATURE REVIEW

Essential Oils

Aromatic plants produce phytochemicals including essential oils (EOs), tannins, flavonoids phenolics and other secondary metabolites (Brenes & Roura, 2010; Gwayali & Ibrahim, 2014; Tohidi et al. 2017). The EOs are produced by aromatic plants for purposes of defense against microbial invasion (Sofos et al. 1998; Calo et al., 2015). These naturally occurring phytochemicals are effective against microorganisms by inhibiting growth and/or reducing the survival of food spoilage and pathogenic microorganisms in different food systems (Callaway et al., 2011; Gyawali & Ibrahim, 2014). The antimicrobial properties exhibited by EOs make them suitable alternatives to traditional chemical food preservatives and additives due to the increasing demand by consumers for foods with more natural additives (Fisher & Phillips, 2008; Solorzano-Santos & Miranda-Novales, 2012; Mazzarrino et al., 2015; de Medeiros Barbosa et al., 2016).

Traditionally, control of food spoilage and pathogenic bacteria has mainly been achieved by using synthetic bactericidal or bacteriostatic chemicals. However, the use of some chemicals to improve shelf life and provide safe food has been associated with carcinogenicity, teratogenicity, acute toxicity and slow degradation leading to environmental pollution (Faleiro, 2011; Swamy et al., 2016). These undesirable outcomes have led to negative public perceptions of synthetic chemical preservatives and generated great interest in the use of natural antimicrobials as substitutes for controlling food spoilage and pathogenic bacteria (Faleiro, 2011; Calo et al., 2015; Swamy et al., 2016).

Natural antimicrobials are naturally occurring substances from living biological systems and which have not been modified in a laboratory or anywhere outside of their biological environment (Li *et al.*, 2011; Sirsat, Muthaiyan, & Ricke, 2009). The emergence of bacterial

resistance to antibiotics has also led to increased interest in EOs as natural alternative agents for control pathogenic bacteria (Burt, 2004; Fisher & Phillips, 2008; Monte et al., 2014).

Essential oils as secondary metabolites of plants

Essential oils are present in various components or parts of plants such as leaves, barks, seeds, flowers, stems and roots (Erasto, Bojase-Moleta, & Majinda, 2004; Rahman & Gray, 2002; Verma & Shukla, 2015). The EOs can be extracted from plants through methods such as distillation, cold pressing or through maceration (Faleiro, 2011; Solorzano-Santos & Miranda-Novales, 2012; Tiwari & Rana, 2015) and are often poorly soluble in water. They are very complex mixtures of hundreds of individual components (Negi, 2012) with the composition of an EO being dependent on what part of the plant is used to extract the EO: bark, leaves, flowers or seeds (Novak, Draxler, Gohler, & Franz, 2005; Kabera et al., 2014). Sofos et al., 1998, suggested that the naturally occurring antimicrobials in plant tissues are a consequence of evolution, as plants synthesized EOs and their components as a defense mechanism against microbial invasion.

The antimicrobial properties of EOs are linked to their bioactive volatile components (Mahmoud & Croteau, 2002). Those EO components can be categorized as terpene or aroma compounds (Bakkali et al., 2008). Chemically, EOs consist of terpene compounds (mono-, sesqui- and diterpenes), alcohols, acids, esters, epoxides, aldehydes, ketones, amines and sulfide (Bakkali et al., 2008; Calo et al., 2015). The yield of EOs can vary with species of aromatic plant, agronomic practices and seasonal changes (Hudaib et al. 2002; Mendonca et al. 2018). Also, different types of environmental and growth stressors exerted on aromatic plants elicit responses that affect the yield and constituents of EOs from the same variety or species of plants (Rebey et al. 2012; Sarikurkcu et al., 2015). For example, in response to water deficiency, Rebey et al. (2012) reported a 1.4-fold increase in EO content in cumin seeds after exposure to a moderate water deficiency but

the EO content decreased by 37.2% in cumin seeds that were exposed to severe water shortage. Methods of handling or processing aromatic plants such as harvesting, drying, extraction and distillation also affect the yield and composition of EOs (Fathi & Sefidkon, 2012; El-Zaeddi et al., 2016). Thus, physical and chemical factors also contribute to variations in yield and composition of EOs extracted from aromatic plants.

Application of Essential Oils in control of Food Spoilage and Pathogenic Bacteria

The demand for natural and clean-label foods by consumers presents a new challenge to the food processing industry as they aim to produce high-quality safe food. This challenge is related to gaining market share via development and sale of food products with added natural preservatives. Although, there are fewer food products available commercially that have been preserved with EOs, numerous studies to evaluate the effectiveness of EOs against spoilage and pathogenic bacteria have been conducted with a variety of foods such as meats, poultry, juices, vegetables and ready-to-eat products (Burt, 2004; Mith et al., 2014; Iseppi et al., 2018). The EOs have exhibited antimicrobial activity against several foodborne pathogens including but not limited to *E. coli* O157:H7, *Salmonella typhimirium*, *Listeria monocytogenes* and *Campylobacter Jejuni* (Callaway *et al.*, 2011; Iseppi et al., 2018).

Goulas & Kontominas (2007) investigated the effects of EOs obtained from oregano, rosemary, thyme, sage, basil, turmeric, coriander, ginger, garlic, nutmeg, clove, mace, savory, and fennel and used them alone or combined with other EOs to extend the shelf life of meat products and they found a significant extension in shelf life in their meat samples. Many EOs consist of multiple major and minor components that may provide a synergistic or additive effect during the inhibition of growth of bacteria to mimic hurdle technology (Gavaric et al., 2015).

García-García, Lopez-Malo, & Palou (2011), reported the minimum bactericidal concentration (MBC) of thymol, carvacrol and eugenol individually and when they were combined against *Listeria innocua.* Individually, the MBC of carvacrol, thymol, and eugenol against *L. innocua* was 150, 250, and 450 mg/kg. Combinations of thymol and carvacrol at concentrations of 62.5 mg/kg and 75 mg/kg respectively and or combinations of thymol and eugenol at concentrations of 56.25 mg/kg and 125 mg/kg completely inhibited the growth of *L. innocua.* The three components carvacrol, thymol and eugenol were combined at the following concentrations 75, 31.25, and 56.25 mg/kg respectively, resulting to complete inhibition of *L. innocua.* Combining two EOs, thyme and orange essential oils, Thannisery and Smith (2014) reported significantly ($P < 0.05$) decreased numbers of *Salmonella* and *Campylobacter* on raw chicken when the two EOs (0.5%) were applied (via vacuum tumbling) in a salt-phosphate marinade solution. Initial numbers of viable *S.* Enteritidis and *C. coli* on broiler breast fillets were reduced by 2.6 and 3.6 log cfu/ mL, respectively. Those same authors demonstrated that the efficacy of EOs against pathogens on raw poultry could be significantly improved when EOs are used in combination. This approach reduces the individual amounts of EO needed to preserve foods with potential for minimizing the sensory changes that can result from application of large doses of EOs to foods.

EOs have shown a great potential during *in vitro* antimicrobial assays; however, generally, a greater concentration of EO is usually necessary to achieve the same antimicrobial effect in food systems (Burt, 2004; Chang et al., 2015). Factors present in complex food matrices such as fat content, proteins, water activity, pH, and enzymes can potentially diminish the efficacy of EOs (Calo et al. 2015). The EOs are more stable and soluble at low pH and therefore exhibit enhanced antimicrobial activity in high acid conditions (Burt, 2004; Franklyne et al., 2019). In this regard, the low pH of acidic marinades for meats may be exploited for enhancing the antimicrobial effect

of essential oils against meat-borne pathogens. Higher salt concentrations and low ambient temperatures have also been reported to enhance the antimicrobial activity of some EOs. Most EOs such as thyme oil and cinnamaldehyde are associated with strong odors, which can significantly alter the organoleptic properties of food resulting from the use of a minimum inhibitory concentration (MIC) dose that would only inhibit bacterial growth rather than the minimum bactericidal concentration (MBC) that kills bacteria.

Mechanism of action of essential oils

The information in this section is not written to give a detailed account of the mechanisms of action of different EOs but rather to provide a general overview on how EOs are believed to cause microbial growth inhibition or kill foodborne microorganisms considering that these mechanisms are still not yet properly understood (Swamy et al., 2016). Essential oils are composed of several components; thus, their antimicrobial activity cannot be solely attributed to only one component. It is well established that the chemical compositions of EOs from plants of the same species could significantly differ based on geographical origin, growing conditions, agronomic practices and harvesting period of the plant (Sakkas & Papadopoulou, 2017). This implies that there is a possibility of variation between batches of EOs and this is enough to cause inconsistencies in the explanations for mechanisms of action of an EO or affect the degree of susceptibility of gram-positive or gram-negative bacteria to EOs (Burt, 2004; Calo et al. 2015).

The EOs and their components are generally characterized as hydrophobic, allowing the EOs to penetrate the cell membrane thus increasing cell permeability (Burt, 2004). The disruption of the cytoplasmic membrane by EO beyond the ability of the cell to repair the damage can result in cell death (Li et al., 2011). Bajpai et al., (2012) attributed the antimicrobial activity of EOs to their ability to diffuse through the cell membrane because of their lipophilic properties into the

cytoplasm of the cell therefore inducing inhibitory activity. The phenolic nature of several EOs has also been associated with disruption of the cell membrane resulting in structural and physiological impairment of the cell such as increased cell permeability, denaturation of ATPases, diffusion of the proton motive force, and increased leakage of cell contents to eventually cause cell death (Bajpal et al. 2012; Li et al. 2011; Sakkas et al., 2017).

Difference in sensitivity of gram-positive and gram-negative bacteria

The antimicrobial effect of EOs is mainly attributed to their interaction with bacterial outer membrane and/or cytoplasmic membrane to inactivate both gram-negative and gram-positive bacteria. Based on the distinctive physiological and phenotypical features on the cell envelope of both gram-negative and gram-positive bacteria, the generally accepted school of thought is that EOs exhibit more bacteriostatic and bactericidal effects against gram-positive bacteria compared to gram-negative bacteria (Sokovic et al. 2010; Markelova & Semenova; 2017). This argument is based on the premise that EOs directly interact with the hydrophobic components of the cytoplasmic membrane of gram-positive bacteria, while gram-negative bacteria are believed to be more tolerant to EOs because they possess a hydrophilic outer membrane (Kim et al. 1995; Semeniuc et al., 2017) which reduces the extent of penetration of hydrophobic compounds into the cell. In this regard, Chorianopoulos et al., 2004 reported that gram-positive bacteria such as *Staphylococcus aureus*, *Listeria monocytogenes* and *Bacillus cereus* were more sensitive to EOs than gram-negative bacteria such as *Escherichia coli* and *Salmonella enteritidis* supporting the argument by Kim et al. 1995. However, Dorman and Deans (2000) reported that thymol and carvacrol acted differently against gram-positive and gram-negative bacteria contrary to other studies. When used in combination, thymol and carvacrol caused damage to the outer membrane of gram-negative bacteria and increased the permeability of the cytoplasmic membrane to result

in release of lipopolysaccharide and leakage of ATP (Burt, 2004). Interestingly, of the gram-negative bacteria Pseudomonads, which mainly cause food spoilage, *P. aeruginosa* appears to be least sensitive to the action of EOs (Dorman and Deans, 2000). The reason for such unusually high resistance of a gram-negative bacterium to EOs requires investigation.

Thyme Oil

Thyme oil is an essential oil (EO) obtained from the thyme plant, *Thymus vulgaris* L. (family: Labiatae or *Laminaceae*), which is indigenous to the Mediterranean region (Spain, Italy, France, Greece, etc.) (Hudaib et al. 2002; Mancini et al., 2015). The thyme plant has for generations been used as an important source of thyme oil and some of its constituents such as thymol, carvacrol, flavonoids, caffeic acid and labiatic acid are derived from the various parts of the plant (Leung & Foster, 1996; Dauqan & Abdullah, 2017). Hudaib et al. (2002) reported that there are different chemotypes of thyme oil from the same species of thyme plants linking the variations in chemical composition to different seasons and the different stages of the plant life cycle. The variations in chemical composition are also influenced by what part of the plant the EO is extracted from, plant growth environment, type of cultivation, and/or storage conditions for the harvested crop or the EO (Guillen & Manzanus, 1998; Gavaric et al., 2015). The pharmacological properties of thyme oil and that of its different extracts and constituents have been thoroughly studied for their industrial application in food systems and medicine in addition to the plant's (herb) traditional uses (Leung & Foster, 1996; Iseppi et al., 2018). The plant species thymus exhibits strong inhibitory effects against bacteria, fungi, viruses and parasitic organisms. The species also show significant antioxidant and spasmolytic activities (Hudaib et al. 2002)

Components of Thyme oil

Different concentrations of thyme oil components have been widely reported by researchers because the chemotypes used in those studies varied according season, stage of maturity of the thyme plant, geographical location and method of extraction (Imelouane et al. 2009; Hudaib et al. 2002; Mohammadi et al., 2018). The components of essential oils including but not limited to terpenes, terpenoids, phenylpropenes and "others", are classified according to the chemical structure of their active functional groups. Major volatile constituents from the thyme plant are geranial, linalool, γ-terpineol, carvacrol, thymol and trans-thujan-4-ol or terpinen-4-ol (Imelouane et al. 2009; Calo et al., 2015). Terpenes are formed in the cytoplasm of plant cells through the mevalonic acid pathway starting with acetyl-CoA (Hyldgaard et al., 2012; Dauqan & Abdullah, 2017) and are formed as several isoprene (C_5H_8) units combine. Caballero et al. (2003) reported that terpene hydrocarbons were arranged into cyclic structures into monocyclic and bicyclic structures by enzyme cyclase. The second major class of active compounds in thyme oil is terpenoids. Through biochemical modification, enzymes add oxygen molecules to terpenes by moving or removing methyl groups to form terpenoids. Carvacrol, thymol, linalool, geraniol, linalyl acetate, citronellal, piperitone, and menthol are examples of terpenoids (Hyldgaard et al., 2012; Golabadi et al., 2018). Other classes of terpenoids include: alcohols, aldehydes, esters ketones, ethers, epoxides, and phenols. The third class is phenylpropenes and shall be discussed in the subsequent section. Table 1 shows the compounds isolated from thyme oil used in the present study and analyzed by gas chromatography mass spectroscopy (GCMS). The two major components of that thyme oil are thymol (51.07%) and o-cymene (24.1%).

Table 1. Results of compositional analysis of thyme oil based on analysis using GC-MS

Compounds	RT	RI_s	RI_{Nist}	Percent (%)
α-pinene	5.98	942.5	937	1.78
Camphene	6.37	956.8	952	0.42
β-Myrcene	7.29	990	991	0.55
o-Cymene	8.24	1024.3	1022	24.1
Eucalyptol	8.45	1032	1032	1.67
γ-Terpinene	9.14	1057.2	1060	5.42
4-Carene/Linalool	10.27	1097.9	1099	4.40
Camphor	11.63	1147	1145	1.49
Isoborneol	12.09	1164	1157	0.14
Endo-borneol	12.32	1172.6	1167	0.73
Terpinen-4-ol	12.56	1181.2	1177	0.72
Thymol	15.57	1293.2	1291	51.07
Carvacrol	15.82	1301.5	1299	3.33
Caryophyllene	18.99	1422.6	1419	0.20
Caryophyllene oxide	22.95	1585.4	1581	0.18

(Kiprotich et al., 2019, unpublished data) RT: Retention time, RI_s: Kovat′s Retention index of thyme oil compounds found in the sample used in this study; RI_{Nist}: Kovat′s Retention index in NIST14 library.

Antimicrobial Activity

The antimicrobial activity of thyme oil cannot be attributed to a single component because of the diverse family of compounds found in this EO. These components exert different pressures on bacterial cells and may exhibit synergistic or additive antimicrobial effects. The antimicrobial effect of thyme oil is attributed to phenolic terpenoids such as thymol, carvacrol, geraniol and linalool (Caballero et al. 2003; Boskovic et al., 2015; Sakkas & Papadopoulou, 2017) that each have a hydroxyl group and delocalized electrons in the benzene ring structure. The oxygen of the hydroxyl group can donate a lone pair electron into the ring structure to cause an increase in electron density and make the ring much more reactive than benzene alone. That high reactivity is responsible for the antimicrobial actions of phenolic compounds including disruption of the cytoplasmic membrane and inhibition of various enzymes such as topoisomerase, NADH-cytochrome c reductase, and ATP synthase (Borges et al., 2015; Cushnie & Lamb, 2011; Tsuchiya, 2015; Verstraeten et al., 2015). Terpenoids have a great potential for application in the food industry because they are broad spectrum antimicrobial agents inhibiting a wide variety of microorganisms of interest in food safety and spoilage.

Compared to terpenoids, terpenes in general do not possess high antimicrobial activity. When they were used against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus cereus*, these key compounds in thyme oil; α-pinene, β-pinene, β-myrcene, p-cymene, β-caryophyllene, and γ-terpinene exhibited little to no antimicrobial effect (Koutsoudaki et al., 2005). Rao et al. (2010) demonstrated a similar trend upon the investigation of the effect of cymene and γ-terpinene against *Saccharomyces cerevisiae* as no significant effect was noted.

Antimicrobial mode of action

Thyme oil, like other plant essential oils, contain numerous components that may independently have several targets in the bacterial cell. This implies that studies pertaining to the antibacterial mode of action of thyme oil are needed to determine the components' target sites, mode of action and how their activity is impacted by environment factors such as pH and temperature. The main constituent in thyme oil is thymol a phenolic monoterpenoid that has some structural similarities with carvacrol, the only difference being the position of the hydroxyl group on the phenolic ring of either compound. (Hyldgaard et al., 2012; Rao et al., 2019). They are both thought to cause structural and functional damages to the cytoplasmic membrane (Calo et al. 2015).

Thymol exhibits strong inhibitory effects against food spoilage and pathogenic bacteria, however, the primary mechanism by which thymol achieves inhibition is still somewhat ambiguous. Some studies link thymol to the disruption of outer and inner cell membranes, damage through interaction with membrane proteins and impairment of intracellular organelles. The interaction with thymol is thought to affect membrane permeability, demonstrated by loss of the charge difference across the cytoplasmic membrane, cell infiltration of ethidium bromide, and cellular leakage of potassium ions, ATP, and carboxy-fluorescein (Walsh et al., 2003; Xu et al., 2008). Helander et al. (1998); Shapira and Mimran (2007), observed the release of LPS and the disruption of the outer membrane when *E. coli* cells were treated with thymol. To further illustrate the mechanism of disruption exhibited by thymol, Helander et al., 1998 treated *E. coli* cells with excess magnesium, however this could not prevent membrane disruption suggesting that thymol did not rupture the outer lipopolysaccharide membrane by chelating cations. Thymol causes alteration to the cell by attaching to the polar head-group region of the lipid bilayer (Helander et al., 1998).

Carvacrol is present in thyme oil but in much lower concentrations compared to thymol, nonetheless it still contributes to the antimicrobial effects of thyme oil. Just like thymol, carvacrol is a phenolic monoterpenoid and a major component of oregano. Carvacrol exerts its antimicrobial effect by positioning itself in the membrane causing an increase in permeability (Hyldgaard et al., 2012). Helander et al. (1998) and La Storia et al. (2011) demonstrated carvacrol's damage to the outer membrane of gram-negative bacteria causing the release of LPS. Although it has been demonstrated that carvacrol disrupts the bacterial outer membrane, there is a school of thought suggesting that exposure of bacterial cells to carvacrol causes passive transport of ions in response to damaged cytoplasmic membranes. The hydroxyl group on the phenolic structure of carvacrol acts as a transmembrane carrier for monovalent cations, carrying H^+ into the cell cytoplasm and transporting K^+ back out across the cell membrane (Ultee et al., 2002; BenArfa et al., 2006). However, this mechanism proposing that carvacrol provided a hydroxyl group that transported protons across the membrane was disputed by Veldhuizen et al. (2006) as it had been earlier suggested by Ultee et al. (2002) and BenArfa et al. (2006). To refute this mechanism, Veldhuizen et al. (2006) demonstrated that the hydroxyl group of carvacrol was not essential for its antimicrobial activity because a non-hydroxyl compound on carvacrol showed higher activity, ruling out the hydroxyl group. Those previous research findings and arguments suggest that the exact mechanisms of action of any EO is challenging to prove with a high degree of scientific certainty.

Cinnamaldehyde

Cinnamaldehyde is a component of the cinnamon essential oil and naturally occurs in the bark of the cinnamon tree. Cinnamaldehyde is responsible for the flavor and odor of cinnamon and makes up 55-75%, 1-8%, and 70-95% of essential oil extracted from cinnamon bark, cinnamon leaf, and cassia, respectively (Ross, 1976). Cinnamaldehyde is traditionally used as flavoring substance and is generally recognized as safe (GRAS) under the Code of Federal Regulations Title 21, part 182. It has multiple applications in fragrances, toiletries and cosmetics (Cocchiara et al., 2005; Firmino et al., 2018). Several studies have shown that cinnamaldehyde exerts strong antimicrobial effects against food spoilage and pathogenic microorganisms (Ooi et al., 2007; Manu et al., 2017; Friedman, 2017).

Chemical structure

Cinnamaldehyde (2E-3-phenlprop-2-enal) is a phenylpropene and belongs to a class of organic compounds called phenylpropanoids, synthesized in plants from an amino acid precursor, phenylalanine. Phenylpropanoids consist of a six-carbon aromatic phenol group and the three-carbon propene tail of cinnamic acid, produced in the first step of phenylpropanoid biosynthesis. Examples of phenylpropenes include; eugenol, isoeugenol, vanillin, safrole, and cinnamaldehyde (Cocchiara et al., 2005; Wang et al., 2016). Upon extraction from cinnamon oil, cinnamaldehyde is commonly referred to as trans-cinnamaldehyde because it consists of a phenyl group attached to an unsaturated aldehyde. Lens-Lisbonne et al. (1987) estimated cinnamon oil contained about 65% of trans-cinnamaldehyde. Cinnamaldehyde (C_9H_8O) has a molecular weight of 132.16 g/mol.

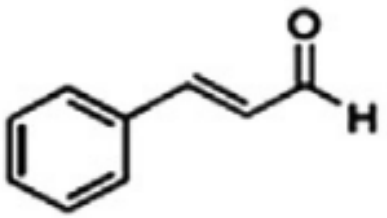

Figure 1; Chemical structure of Cinnamaldehyde. (Adopted from Jayasena & Jo, 2013)

Antimicrobial activity and mode of action

The aldehyde group of cinnamaldehyde covalently bonds with cellular DNA and proteins in an adjacent manner, interrupting normal cell physiological functions (Feron et al., 1991). However, this mechanism is still largely inconclusive as cinnamaldehyde exhibits divergent antimicrobial properties at different concentrations or doses when applied to bacterial cells. Low concentrations of cinnamaldehyde inhibits enzymes involved in cell division and at sub-lethal concentrations, it is an ATPase inhibitor whereas, at lethal concentrations, it disrupts the cell membrane (Kwon et al., 2003; Nowotarska et al., 2017). Cinnamaldehyde inhibited cell division in *B. cereus* as a mode of action by preventing cell separation even when the septa were present (Kwon et al., 2003). Cinnamaldehyde prohibited cell division by binding the FtsZ protein that is focal in GTP dependent polymerization and also essential in the formation of a contractile ring function at a site for future cell division (Domadia et al., 2007; Hemaiswarya et al., 2011).

At sub-lethal concentrations, Helander et al. (1998), demonstrated that cinnamaldehyde entered the periplasm and possibly the cytoplasm where it inhibited the activity of transmembrane ATPase and did not affect the outer membrane of *E. coli*. To confirm access of cinnamaldehyde to the cytoplasm, Gill and Holley (2006a, b) demonstrated lowered ATPase activity of isolated cell membranes at increasing levels of cinnamaldehyde (13.6–1362μg/mL). However, the

concentration of cinnamaldehyde that was required to inhibit ATPase disrupted the cell membrane of *E. coli* (681–1362μg/mL) thus ATPase inhibition could not be the primary cause of cell death (Gill and Holley, 2006a). Upon exposure to cinnamon oil containing 73% cinnamaldehyde, *P. aeruginosa* exhibited elimination of the charge difference across the cytoplasmic membrane, loss of membrane integrity, lowered cellular respiration and coagulation of cytoplasmic material while *S. aureus* cells entered a viable but non-culturable state (Bouhdid et al., 2010).

Bacteria Life Cycle

Like most living organisms, bacteria undergo different phases of physiological, phenotypical, genetic and morphological transformation over time. Conventionally, the bacterial life cycle is described by four different phases namely, lag, exponential, stationary and death phase (Wang et al., 2018). Those four phases are determined by the availability of nutrients and conduciveness of the environment to growth of bacterial cells (Roszak and Colwell, 1987; Maier & Pepper, 2015). The lag phase of the bacterial life cycle can be characterized as a period of adaptation of bacterial cells when they are introduced to a new medium or environment and they thus exhibit low rates of cell division (Baranyi, 2002; Adkar et al., 2017). The lag phase is a period of physiological and metabolic adaptation, whereby cells produce new or modify existing "tools" such as enzymes, organelles, membranes and or proteins to facilitate survival in the new environment (Pin and Baranyi, 2008). Upon adaptation to the new environment, the cells utilize nutrients and then rapidly divide through binary fission giving rise to an exponential phase (Kolter et al. 1993). The exponential phase of the bacterial life cycle is largely dependent on the availability of nutrients, which is a limiting factor to cell growth. Consequently, when the actively multiplying cells deplete the available nutrients over time they transition into the stationary phase (Navarro Llorens et al., 2010). At the stationary phase, stress factors such as accumulation of metabolic

waste and nutrient exhaustion set in, reducing the rate of cell division. This is followed by death of cells and this phase is referred to as the death phase (Delpy et al., 1956; Flemming et al., 2016). The information in this section of this literature review will focus on stationary phase cells and the cells that survive after the death phase has occurred as these phases play a key role in transmission and survival of foodborne spoilage and pathogenic bacteria.

Stationary phase cells

The stationary phase can be described as a nutrient-limiting condition for bacterial cells as cells will theoretically remain in the exponential phase provided, they receive a constant supply of sufficient nutrients to fully support the cell population (Navarro Llorens et al., 2010; Chubukov & Sauer, 2014). The transition of bacterial cells from exponential to the stationary phase is a survival strategy that allows cells to endure nutrient depletion and accumulation of metabolic waste resulting from overpopulation or when the growth medium reaches its maximum carrying capacity (Kolter et 1993; Jaishankar & Srivastava, 2017). Physiologically, cells transitioning from the exponential phase into the stationary phase can be viewed as undergoing a preparatory stage for response to stress be it nutrient depletion or accumulation of waste and thus allows the cells withstand adverse conditions (Finkel, 2006).

The entrance into the stationary phase is a highly regulated process and involves modifying and regulating gene expression to provide bacterial cells with the necessary metabolic “tools” to survive harsh and stressful conditions (Cases et al., 2010). Environmental stress factors such as accumulation of toxic waste, desiccation or unfavorable temperatures provide the necessary signals to transcription regulators and sigma factors that lead to the expression of genes to enhance survival of bacteria under stressful conditions (Gruber and Gross, 2003; Jõers & Tenson, 2016). The genes responsible for the onset of the stationary phase are synonymous with those involved

with response to stress. The stringent response phenomenon has not only been described as a response to stress factors such as starvation but also as a mechanism that leads to the onset of the stationary phase (Jõers & Tenson, 2016). This involves the downregulation of nucleic acid (RNA and DNA) biosynthesis, DNA replication and cell division when cells are deprived of amino acids (Magnusson et al., 2005).

Bacterial death phase

Depletion of nutrients and accumulation of metabolic waste eventually leads to death of cells in the stationary phase. Bacterial cell death can be described as loss of viability caused by degradation of cellular macromolecules, membranes and proteins because of oxidative damage (Dukan and Nystrom, 1998). However, some scholars suggest that bacterial death phase may not just be because of oxidative damage but rather a strategic survival mechanism that purposefully allows 90-99% of the bacteria to die to provide nourishment to the surviving population in a nutrient-depleted environment (Danial and Korsmeyer, 2004; Allocati et al., 2015).

Bacterial cell death may also be explained through the toxin-antitoxin (TA) mechanism that is responsible for post-segregational killing (PSK) of daughter cells to maintain cell population during the late stages of the stationary phase (Engelberg-Kulka et al., 2006; Otsuka, 2016; Hall et al., 2017). The TA system consists of two genes with one gene encoding for a toxin and the other (located on a plasmid) encoding for an antitoxin. Those genes are acquired hereditarily by daughter cells during cell division (Gerdes et al., 1986; Harms et al., 2018). The TA system works in a way that daughter cells that inherit the plasmid during cell division survive as those that do not are killed because cells lacking plasmids cannot produce antitoxin to neutralize the stable toxin (Bravo et al., 1987; Otsuka, 2016). However, the role of the TA system is fully not understood as scholars such as Amitai et al., (2009) argue that this deliberate killing of some of the bacterial cells

is to provide nourishment to the surviving population under conditions of starvation. Gerdes et al. (2005); Wen et al. (2014) postulate that the TA system sets a preparatory stage for substantially reducing metabolic activity and inhibiting growth to allow the bacterial cells survive harsh conditions.

Long-term survivor phase

It has been established that 90-99% of a population of bacterial cells in a batch culture die at the death phase supplying nutrients from their debris to the surviving population of cells, which can retain their viability for months or years suggesting a fifth phase in the bacteria life cycle (Finkel, 2006; Wen et al., 2009; Wen et al., 2013). The long-term survival (LTS) phase has been described by Finkel (2006) as an extension of the stationary phase occurring after the death phase. The transition to the LTS phase is characterized by significant physiological, metabolic and phenotypical changes in cells.

The transition of stationary cells to the LTS phase is directly linked to the prevalence of stressful conditions such as starvation, nutrient depletion or accumulation of metabolic waste surrounding bacterial cells (Navarro Llorens et al. 2010). The LTS phase is characterized by very slow growth rates, reduced protein synthesis, low biosynthesis of nucleic acids and reduced active transportation of nutrients (Reeve et al. 1984; Brown et al., 2010). The changeover of stationary phase to LTS phase is associated with significant morphological changes in the cells through dwarfism and reductive division that lead to formation of small, spherical cells (Nystrom, 2004; Pechter et al., 2017). The cells achieve dwarfism through a type of “self-digestion” similar to autophagy in eukaryotic cells, whereby eukaryotic cell organelles and structures such as membranes are degraded (Nystrom, 2004; Takano et al., 2017). Wen et al. (2009) observed a

morphological change in *L. monocytogenes* cells from rods (exponential and stationary phase cells) to coccoid cells when those cells were in the LTS phase.

Tolerance of LTS cells to antimicrobial interventions and environmental stress

The transition of bacteria cells from the exponential phase to stationary phase and finally to the LTS phase is basically a survival mechanism that allows these bacteria to survive in harsh and stressful environments and to also cope with extreme energy limitations linked to nutrient exhaustion (Hoehler and Jorgensen, 2013). Wen et al. (2009) demonstrated that LTS cells of *L. monocytogenes* had a higher tolerance to heat and high pressure compared to exponential and stationary phase cells. Furthermore, Wang et al. (2018) showed that LTS cells were more tolerant to UV radiation compared to stationary and exponential phase cells of *S. typhimirium* in apple juice.

Environmental stress influences dividing cells to enter the stationary phase where a build-up of a sigma factor Rpos occurs even in the LTS phase (Hengge-Aronis, 2002). The genes that are expressed by the sigma factor Rpos provide bacteria with various stress resistance mechanisms against heat shock, oxidative stress, osmotic stress and pH changes (Weber et al., 2005; Chib & Seshasayee, 2018). The cells in the in the stationary and LTS phase also express BolA, a protein in charge of regulating transcription of genes that produce penicillin-binding proteins PBP5 and PBP6 conferring these cells tolerance to penicillin (Santos et al. 2002). Gram-negative bacteria in the LTS phase produce cell envelopes modifying their outer membrane, periplasm and peptidoglycan as a protective barrier and this is achieved by impregnating the exterior surface of the outer membrane with lipopolysaccharides (Huisman et al. 1996). For protection against osmotic stress, the cells build-up an oligosaccharide, trehalose, in the periplasm (Huisman et al. 1996). Another protective mechanism occurs in the cytoplasm, whereby the nucleoid is condensed

for purposes of protecting the DNA material (Wen et al., 2009). All these changes occurring in LTS phase cells confer these cells with a better ability to withstand antimicrobial interventions and environmental stress compared to exponential and stationary phase cells.

Food safety implication of long-term survivor cells

Traditionally, the design and implementation of antimicrobial interventions such as temperature-time regimes, high pressure or radiation doses are based on and authenticated by their ability to kill stationary phase cells (Wang et al. 2018). However, pathogens that contaminate our food supply can come from environmental sources. In the natural environment, bacteria spend most of their life cycle in the LTS phase because of constant exposure to harsh conditions that are not conducive to growth. Therefore, those conditions necessitate that cells survive until they encounter favorable conditions. There is a growing body of research demonstrating the very high resistance of LTS phase cells (compared to stationary phase cells) of pathogenic bacteria to physical antimicrobial processes (Wen et al., 2009; Wang et al., 2018). Therefore, antimicrobial interventions tested against stationary phase cells may not be able to adequately inactivate LTS cells in foods, making it unsafe for human consumption or vulnerable to spoilage (Hawkins et al., 2019).

Listeria monocytogenes

Listeria monocytogenes is a foodborne pathogen of major concern to the food processing industry especially in the production of ready-to-eat products and causes listeriosis in humans (Wen et al., 2009; Ferreira et al., 2014). *L. monocytogenes* can be characterized as a small rod-shaped gram-positive bacterium that forms no spores and can grow in the absence or presence of oxygen. This pathogen can grow under refrigeration temperatures and express β-hemolysin that causes destruction of red blood cells (Farber and Peterkin, 1991). *L. monocytogenes* is a catalase

positive bacterium and oxidase negative implying that it does not produce certain cytochrome c oxidases.

Scallan et al. (2011) estimated that 1600 people in the United States suffer from a foodborne illness associated with food contaminated with *L. monocytogenes,* with a reported mortality rate of 19% although this varies amongst people of different age groups and physiological status. Immunocompromised persons such as the elderly, neonates, pregnant women and infants reported a higher mortality rate because their T-cell mediated immunity is suppressed (Farber & Peterkin, 1991; Swaminathan & Gerner-Smidt, 2007; Lomonaco et al., 2015).

Sources of contamination

L. monocytogenes is an environmental contaminant and has been isolated from water, soil and or animal droppings and is therefore ubiquitous. The natural habitat for *L. monocytogenes* is decaying vegetation. The most common vehicle for transmission of this pathogen is through consumption of contaminated food or through cross-contamination from food processing surfaces to food (Luchansky et al. 2017). *L. monocytogenes* is a hardy microorganism and has shown tremendous ability to withstand extreme conditions of cold such as freezing, desiccation and heat compared to other non-spore formers (Farber and Perterkin, 1991; Camargo et al., 2017). The foods that have been implicated in the transmission of this pathogen are mainly ready-to-eat foods that do not undergo a heating step prior to consumption for instance fermented sausages, deli meats, ice cream, milk, cheeses and smoked meats (Zhu et al., 2017).

Disease outbreaks associated with apple juice

There is an increasing demand for minimally processed fresh juices today that are devoid of synthetic chemical preservatives and have been minimally treated by heat thus increasing consumer risk of infection by a foodborne pathogen (Burnett and Beuchat, 2001). There has been

no published report on a disease outbreak that resulted from contamination of apple juice by *L. monocytogenes.* However, this pathogen is ubiquitous in the environment and therefore can contaminate fruits and consequently raw fruit juices. The *L. monocytogenes* can survive and growth of in fruit juices (Yuste and Fung, 2002; Ingham et al., 2006; Buchanan et al., 2017) and has been isolated from apple juice, apple cider and a blend of apple and raspberry (Sado et al., 1998; Angelo et al., 2017). Therefore, there is a potential for contaminated juices or cider to transmit *L. monocytogenes* to humans (Ewing et al., 2018). Unpasteurized apple juice has been implicated in numerous foodborne outbreaks as people have fallen ill from consuming apple juice contaminated with other pathogens including *E. coli* OH157: H7 and *Salmonella* (Burnett and Beuchat, 2001; Park et al., 2017).

Salmonella enterica

Salmonella enterica is one of the two species of bacteria that make up the genus *Salmonella* that belongs to the family *Enterobacteriaceae* (Hammack, 2012). Phenotypically, salmonellae are rod-shaped, test negative when gram-stained, non-spore-forming and possess flagella (Hammack, 2012). The *S. enterica* is further divided, taxonomically, into six sub-species that currently contain over 2,500 serovars. Those six sub-species are *S. enterica* subsp. *enterica* (I*), S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizonae* (IIIa), *S. enterica* subsp. *diarizonae* (IIIb), and *S. enterica* subsp. *houtenae* (IV), *S. enterica* subsp. *indica* (VI) (Hammack, 2012). Salmonellae are chemoorganotrophic bacteria, obtaining their energy from oxidation of organic electron donors in nature and are facultative anaerobes (Fabrega and Vila, 2013; Yeryomina et al., 2017). They are either invasive (typhoidal) or non-invasive (non-typhoidal) depending on how the symptoms of infection manifest in humans (Okoro et al. 2012). Non-typhoidal *Salmonella* cause an illness called salmonellosis and is the most prevalent foodborne illness worldwide with the CDC reporting an

estimated 1.2 million infections every year. Hammack (2012); Borewicz et al. (2015), reported the mortality rate of patients diagnosed with salmonellosis to be at 1% for healthy individuals and higher at 3.6% in individuals not at optimum health conditions such as those in nursing homes or those who are immunocompromised. Also, typhoidal salmonellae (*S. typhi* and *S. paratyphi*) cause typhoid fever in only humans and higher primates (Okoro et al. 2012; Borewicz et al., 2015) and have a higher mortality rate estimated at 10% in the United States according to estimates from Hammack (2012). In typhoid fever, the pathogens invade the spleen, lymphatic system, kidneys and the blood stream from the small intestine. They produce endotoxins that affect the vascular, neural and water-salt regulation systems of the body leading to septicemia and or hypovolemic shock and if untreated, death (Okoro et al., 2012). Hypovolemic shock is a condition in which excessive loss of blood or fluids (blood plasma) decreases the heart's ability to pump adequate amounts of blood throughout the body. Consequently, this condition results in multiple organ failure (Wang et al., 2013).

Sources of contamination

Salmonella colonizes the intestinal tracts of vertebrates, reptiles, domestic or wild animals, birds and humans. Infected animals and humans can be in an asymptomatic carrier state as they transmit the pathogen to the environment through excretion of fecal matter making *Salmonella* ubiquitous in nature (Hammack, 2012). Once in the environment, *Salmonella* then contaminates our food and water sources, which are then ingested by humans and animals to continue the cycling of that pathogen. Foods of animal origin such as meats, poultry, eggs, milk and dairy products, fish, and shrimp have been traditionally associated with the transmission of *Salmonella*, although foods of plant origin such as fruits (dry and fresh) and vegetables are also recognized as vehicles for this pathogen (Bouchrif et al., 2009; Hammack, 2012; Antunes et al., 2016). Apart from direct

contamination via animal feces or inadequately composted animal manure, the use of contaminated irrigation water plays a critical role in contamination of a variety of fresh produce with *Salmonella* (Steele & Odumero, 2004; Levantesi et al., 2012; Santiago et al., 2018). Another critical source or method of transmission of *Salmonella* is cross-contamination that essentially occurs when the pathogen is spread from a point initially contaminated such as a food, an animal, processing or food handling surface or an in infected human to other foods or surfaces (Jimenez et al. 2008). Cross contamination has been largely linked to handling of infected and asymptomatic pets including dogs, cats, tortoises, lizards, chameleons or frogs and their feces (Bouchrif et al., 2009).

The complexity of the current global market of food involving tons of imported produce and spices from other countries has exacerbated the incidences of produce contaminated with *Salmonella.* This is because the implementation of HACCP and Good Agricultural Practices (GAPs) is not as efficient in developing countries compared to developed nations with improved and superior infrastructure and human resources to control and reduce the transmission of foodborne pathogens such as *Salmonella*. (Rahmat et al., 2016; Hanlon et al., 2018).

Poultry meat as a major source of salmonellosis

Poultry including chickens, ducks, geese and turkeys are epidemiologically important reservoirs of the non-typhoidal strains of *Salmonella* from which widespread contamination of the environment, water and food originates (Antunes et al., 2016). The numerous animal reservoirs for *Salmonella* and the ability of this pathogen to successfully colonize the intestinal tract of healthy poultry and not result to any noticeable illness or symptoms increase the risk of transmission of the pathogen to humans (Hugas and Beloeil, 2014). Horizontal and vertical transmission of *Salmonella* occurs during production where infected but asymptomatic birds infect other healthy birds and their eggs (Cosby et al., 2015). Arsenault et al. (2007) reported that over

50% of the poultry flocks in Canada in that year were infected with *Salmonella* and were asymptomatic.

The bacterial cells in the gut of the bird can then be transmitted to the carcass during processing steps such as evisceration and chilling (Carrasco et al. 2012). Chilling is an important growth inhibitory step for *Salmonella* during poultry processing and is aimed at inhibiting proliferation and spread of the pathogen in the chill tank. The chilling water is usually changed frequently to avoid accumulation of the pathogen; however, the carcasses are packed closely, facilitating cross contamination of carcasses (Sarlin et al. 1998). The infected carcass if undercooked could result in salmonellosis or could contaminate other utensils, surfaces or other foods in the household consequently leading to infection (Arsenault et al., 2007). Jimenez et al. (2008) demonstrated the ability of *Salmonella* to detach from chicken skin and contaminate plastic cutting boards thus increasing the risk of cross contamination associated with contaminated poultry meat.

Numerous outbreaks of salmonellosis have been associated to consumption of poultry meat worldwide resulting to millions of illnesses, hundreds of thousands of hospitalized patients and thousands of deaths (Majowicz et al., 2010).

Sub-lethal Injury

During processing, a food product is usually subjected to heat, high pressure, irradiation, electric fields or microwave energy as a physical means to kill both pathogenic and or spoilage microorganisms. Chemicals, synthetic or natural, such as organic acids, phenols, sorbates, benzoates and essential oils can also have a bactericidal effect that reduces microorganisms until a number that is incapable of spoiling the food or causing harm to the consumer is achieved (Hurst et al., 1976; Wu, 2008; Luz et al., 2014). Those previously stated treatments exert significant

amounts of stress physically or chemically on bacterial cells damaging key cellular structures and/or impairing physiological functions. Depending on the extent of cellular damage, death of all or part of a population of microorganisms in a food can occur. However, a small or large section of the cells might be sub-lethally injured and depending on the intensity or magnitude of the treatment applied and location of the cells in a food matrix (Russel, 1984; Wu et al., 2001a; Wang et al., 2017).

Sub-lethal injury in microorganisms occurs on a spectrum, with cells exhibiting different effects of the treatment even when they are exposed to the same treatment at the same intensity and time (Wesche et al. 2009; Thomas-Popo et al., 2019). Therefore, within a population of sub-lethally injured cells the injury can range from mild to severe. Sub-lethally injured cells can have damaged cell membranes, broken DNA, denatured enzymes or even a dysfunctional membrane transport system leading to leakage of cytoplasmic contents (Hurst, 1977; Noriega et al., 2014). In instances when enzymes such as catalase and superoxide are denatured by heating, the sub-lethally injured cells suffer from oxygen toxicity and are unable to grow in non-selective media under aerobic conditions (Mendonca and Knabel, 1994). Sub-lethally injured cells are likely to go undetected when conventionally plated on selective media. Those cells might repair their injuries and resume normal physiological and metabolic functions, under favorable conditions thus posing a huge threat to food safety (Straka and Stokes, 1959)

Microbial stress leading to sub-lethal injury

An antimicrobial intervention aimed at inhibiting growth or causing death of a bacterial cell can at times cause stress which can compromise the physiological functions of the cell, sometimes causing physical injury (Wesche et al., 2009). Most mechanisms of action of different

treatments inflict stress on the cells at different intensities ranging from mild to severe and when sustained, the stress can lead to death of the cell (Storz and Hengge-Aronis, 2000).

Physical and chemical antimicrobial interventions are sources of bacterial stress and can lead to sub-lethal injury of microbial cells if lethality is not achieved. Extreme and acute changes in pH can result when the pH suddenly drops and causes transmembrane movement of hydrogen ions into the cell. This in turn lowers the pH of the cytoplasm, denatures proteins and enzymes to causes death or injury of the cell (Abee and Wouters, 1999). Also, extreme alkaline conditions could also stress the cells to cause sub-lethal injury or death. In fact, the antimicrobial mechanism of high pH against Gram-negative pathogens involves disruption of the cytoplasmic membrane (Mendonca et al 1994). In this respect, the inability of sub-lethally injured cells to repair damage to their cytoplasmic membrane can lead to cellular death. To demonstrate sub-lethal injury in *S. typhimirium*, Dickson and Siragusa (1994) washed or sprayed beef carcasses with 1% lactic acid, 1% acetic acid and distilled water. Those same authors reported that there was a significant rise in injury (38%) in the surviving populations of *S.* Typhimurium on carcasses that were sprayed with either lactic or acetic acid compared to those sprayed with distilled water only.

An example of a physical means to control microbial growth is by using either very low temperatures (freezing and refrigeration) resulting in cold stress or by use of high temperatures above the optimum growth temperature of the bacteria (Miller et al., 2000; Foster, 2000; Wang et al., 2017). Refrigeration temperatures deliver a cold shock to microorganisms. This cold shock is characterized by slow or no cell division and reduced protein synthesis; however, this condition can change when the cells adapt to their new environment (Van Schothorst and Duke, 1984). Sub-lethal injury under refrigeration temperatures leads to mild degrees of injury (Van Schothorst and Duke, 1984). However, freezing results in moderate to severe degrees of injury compared to

refrigeration since freezing reduces water available for biochemical reactions thus the concentration of solutes to cause osmotic stress on the cells. Also, crystals of ice formed during freezing can result in physical injury to the bacterial cells (Mackey, 1984). Freezing is however considered to be bacteriostatic as it inhibits proliferation of spoilage or pathogenic microbes but does not always lead to death and therefore is not considered as a kill-step during food processing. On the other hand, higher temperatures above the optimum required for bacterial growth might exert stress on bacterial cells, causing injury and ultimately death of the cells (Wesche et al. 2009).

The degree of lethality of a temperature treatment is dependent on intrinsic factors of the food such as water activity, pH, redox potential, the availability of nutrients and antimicrobial substances. Extrinsic factors such as temperature and relative humidity are also important factors to consider when studying the sensitivity of a microorganism to heat because they can also influence the degree of sub-lethal injury in microorganisms (Busta, 1976; Bunning et al., 1990; Farber and Pagotto, 1992). Cells undergo multiple physiological and metabolic changes when they are sub-lethally injured as the cell membranes are often damaged, ribosomal DNA and RNA might endure breaks, and respiratory enzymes might be denatured or inactivated (Ray, 1979; 1992).

Repair of sub-lethally injured bacteria

Sub-lethal injury in bacterial cells is characterized by cells that have lost the ability to proliferate and form visible colonies on selective agars and this form of injury can be classified as metabolic or structural (Wesche et at. 2009; Wu, 2008). Metabolic injury in bacterial cells is also observed when cells fail to form visible colonies on agar with minimal nutrients (Gilbert, 1984). In structural injury, the cells fail to multiply in media which contain selective agents such as bile salts, sodium chloride, crystal violet or antibiotics yet these substances show no effect (inhibitory or lethal) on normal (non-injured) cells (Hurst, 1977; Gilbert, 1984; Hurst; 1984). After an

antimicrobial intervention or stress, the difference in the number of cells (CFU/mL) that only grow on selective media and those that grow on non-selective media represent the number of injured cells within the surviving population (Brashears et al., 2001; Wu, 2008).

Sub-lethal injury occurs on a spectrum ranging from mild to severe, however, just like any other living organism, bacterial cells that are sub-lethally injured might be able to repair their damaged membranes, repair DNA and RNA, synthesize new proteins and enzymes and regain an optimal physiological and metabolic state. This resuscitation of bacterial cells allows them to resume growth and multiplication once favorable conditions such as, but not limited to, pH, nutrients, moisture, optimum temperature and time are provided (Busta, 1976; Hurst, 1984; Jay et al; 2005). The repair of sub-lethally injured cells can be described as a restoration process that allows the cells to replenish cytoplasmic constituents such as enzymes, protein, pH and all components that might have leaked out of the cell (Busta, 1976). Sub-lethally injured bacterial cells resulting from exposure to heat or irradiation that damage DNA and RNA also have to repair damaged nucleotides during this process (Jay et al. 2005). Irrespective of the type of stress or antimicrobial intervention a vegetative cell is exposed to, resuscitation of the sub-lethally injured cells occurs once the cells are supplied with the correct type of nutrients, an environment conducive for growth including optimum conditions of temperature (Ray and Adams, 1984). Once the cells are fully repaired and have regained normal functioning, they can again exhibit resistance to selective agents in selective media. Therefore, they can be grown and enumerated on selective media as physiologically normal cells without any form of sub-lethal injury (Ray and Adams, 1984). Scholars such as Wesche et al. (2009) have suggested that repair in sub-lethally injured cells occurs during the lag phase and therefore the length of the lag phase could be used to estimate the time the cells take to repair in non- selective media.

Recovery and enumeration of sub-lethally injured bacteria

The inability of sub-lethally injured cells to grow on selective media using conventional plating techniques makes detection and enumeration of these cells in a food matrix difficult during routine microbial analysis in a food processing plant. Consequently, this situation is of a great food safety concern to the industry and public safety officials. Sub-lethally injured cells can only grow on non-selective media and therefore particular pathogens of interest cannot be isolated from the general microbial population because selective agars contain selective agents do not allow for repair of injured cells (Ray, 1979; Wu and Fung, 2001).

There are two main methods used for recovering injured bacterial cells namely the liquid repair methods and solid repair media repair methods with the former being used for determination of the most probable number (MPN) and indicator microbes while the solid repair method can be directly used to enumerate sub-lethally injured cells (Ray, 1979; Ray and Adams, 1984). However, for this thesis, the focus shall be on the solid repair methods with specific reference to the thin agar layer (TAL) method.

The thin agar layer (TAL) method was developed by Kang and Fung (1999) and it entails overlaying set selective agar with melted non-selective agar such as TSAYE. The injured cells are then surface plated on the solidified double-layered medium. The non-selective agar provides important nutrients while shielding injured cells from selective agents in the selective medium (bottom layer), allowing them to resuscitate and repair their injuries. Over time, the injured cells repair, the selective agents from the selective agar then migrate to the surface of the agar making the agar selective for the target pathogen (Kang and Fung, 1999; Wu and Fung, 2001; Wu et al. 2001a). Another merit of this method is that hot agar does not come in contact with the injured cells unlike the pour plate method and the surface-overlay plating method, thus reducing incidences of further damaging or killing the sub-lethally injured cells (Wu, 2008). Utilizing the

TAL method, Wu and Fung (2001) and Wu et al. (2001a, b) recovered significant numbers of survivors of *E. coli* O157:H7, *Listeria monocytogenes*, *S. Typhimurium*, *S. aureus*, and *Yersinia enterocolitica* that were sub-lethally injured with heat, acid or cold.

Implication of sub-lethally injured cells in food safety

The ability of sub-lethally injured cells to go undetected on selective media presents an incredible food safety threat because most food processing industries do not use a resuscitation step during routine microbial analysis to allow for the recovery of injured cells. Also, there have been reports by Lou and Youseff (1996) associating previously sub-lethally injured cells with increased tolerance to stresses or antimicrobial interventions following resuscitation. Wesche et al., 2009 also reported increased levels of virulence for sub-lethally injured cells upon resuscitation making their presence in food of greater concern. Besides, some foodborne pathogens have very low infectious doses and therefore it is paramount that all the pathogens are detected, especially the sub-lethally injured cells even if they are in relatively low numbers (Noriega et al., 2014).

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CHAPTER 3. SURVIVAL AND SUB-LETHAL INJURY OF *SALMONELLA ENTERICA* IN ARTIFICIALLY INOCULATED RAW CHICKEN BREAST MEAT MARINATED IN LEMON JUICE WITH ADDED THYME OIL AND YUCCA EXTRACT

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Abstract

Enteric pathogens such as *Salmonella enterica* can survive under conditions of extremely low pH to pose a food safety threat during the process of marinating poultry products. A study was conducted to investigate the survival of *Salmonella enterica* artificially inoculated on chicken breast meat and marinated in lemon juice (L) with added thyme oil (TO) and yucca extract (YEX). Raw chicken breast fillets were artificially inoculated with a five-strain cocktail of *S. enterica* serovars (~10^7 CFU/mL) and immersed for 2, 4, 6, and 8h in four lemon-based marinades at 23°C: lemon juice alone (L), lemon juice with added 0.5% yucca extract (L+Y), lemon juice with 0.5% yucca extract and 0.5% thyme oil (L+Y+0.5%TO) and lemon juice with 0.5% yucca extract and 1.0% thyme oil (L+Y+1.0%TO). Survivors were enumerated by surface plating 0.1mL aliquots of chicken homogenates on xylose-lysine tergitol-4 (XLT4) agar and XLT4 agar overlaid with a thin layer of tryptic soy agar supplemented with 0.6% (w/v) yeast extract (TSAYE) and colonies enumerated after incubation at 35°C for 48 h. All treatments reduced the number of survivors of *S. enterica* after 8h of marination. Marinade solutions containing YEX and TO significantly reduced number of viable cells inoculated on the chicken breast fillet after 8h compared to the control marinade solutions containing lemon juice alone and lemon juice with YEX ($P<0.05$). The L and L+Y decreased cell viability by 2.25 and 2.46 Log CFU/sample respectively after 8h of marination compared to L+Y+0.5%TO and L+Y+1.0%TO that decreased populations of viable cells by 3.58 Log and 4.81 Log CFU/sample after 8h. There was a significant difference in the number of survivors plated on both TAL (XLT4 with overlaid with TSAYE) and XLT media ($P<0.05$). However, there was no significant level of sub-lethal injury caused by the treatments ($P>0.05$). Based on these results, thyme oil has good potential to enhance the antimicrobial efficacy of marinades used for raw poultry meat.

Introduction

Non-typhoidal *Salmonella enterica subsp. enterica* serovars are the most commonly implicated pathogens in foodborne disease outbreaks and the leading cause of bacterial foodborne illnesses in humans worldwide (Bajpai *et al.*, 2012; Boore *et al.*, 2015;). Although the implementation of successful *Salmonella* control programs has reduced the number of human salmonellosis cases involving poultry meat over the past years (CDC, 2011, 2014, 2015a, 2015b), consumption of contaminated poultry products including poultry meat has been largely responsible for numerous salmonellosis outbreaks worldwide (Mead *et al.*, 2010; Finstad *et al.*, 2012). In the United States *Salmonella enterica* cause approximately 1 million infections, 19,336 hospitalizations and 378 deaths annually. (Scallan *et al*., 2011). *Salmonella* frequently inhabit the intestinal tract of poultry (Smith et al., 2007); therefore, a major source of *Salmonella* contamination during processing of poultry is spillage of intestinal contents during the evisceration step. Furthermore, the ability of *Salmonella* to survive under harsh environmental conditions in poultry processing facilities and equipment increases the incidence of cross contamination to previously non-infected carcasses (Carraminana *et al.,* 1997). Due to numerous opportunities for microbial contamination in poultry processing, multiple pathogen control strategies and intervention kill steps are necessary to ensure microbial safety of poultry meat from farm to consumer (White *et al*., 1997). In this regard, antimicrobial marinade formulations might have good potential for use as an intervention strategy to reduce enteric pathogens on raw poultry meat.

Marination involves the soaking or pre-incubation of raw meats in an emulsion or water-based solution containing salt, acids, spices and different aroma additives (Bjorkroth, 2005; Quelhas *et al.,* 2010) with aim of improving tenderness, juiciness, yield, and flavor (Alvarado & McKee, 2007; Pathania *et al.,* 2010). Smith and Acton (2001) estimated that more than 50% of raw poultry may be marinated prior to consumption; therefore, marination presents an ideal

opportunity to exploit the antimicrobial activity of certain aromatic components of herbs and spices against meat-borne pathogens. Low pH marinades containing organic acids have exhibited antimicrobial properties enhanced by active antimicrobial compounds found in different spices and herbs that are utilized for flavoring purposes (Bremer & Osbourne, 1995; Pathania *et al.*, 2010). Also, the addition of plant essential oils (0.5% thyme oil and orange oil combined) to a marinade (water, salt, and phosphate) applied to broiler breast fillets and wings via vacuum tumbling, significantly reduced ($P < 0.05$) numbers of viable *Salmonella* Enteritidis by 2.6 and 2.3 log cfu/ mL of poultry meat rinsate from breast and wings, respectively (Thanissery and Smith, 2012).

Essential oils (EOs) are oily and highly volatile extracts of aromatic plants that exhibit potent antimicrobial activity (Burt, 2004). The oily (hydrophobic) characteristic of EOs is a major impediment to their application in largely water-based (hydrophilic) marinades that may consist of water, salt and phosphate or mainly citrus juices such as lime or lemon juice. The EOs are not miscible in water-based solutions and therefore, require the addition of a surfactant for their solubilization (Mendonca *et al*., 2018; Samperio *et al.*, 2010; Thomas-Popo *et al*., 2019). Considering the increasing consumer demand for more natural alternatives to synthetic food additives, natural surfactants such as Yucca extract from the *Yucca schidigera* plant are gaining much attention from food processors. Yucca extract has FDA GRAS status and is approved for use as ingredients in foods and beverages (Code of Federal Regulations 21CFR 172-510, FEMA number 2973).

While there is a growing body of knowledge on the potential application of EOs as antimicrobials in various food products, published reports on the incorporation of EOs in low pH marinades for pathogen control in poultry meat are scarce. To our knowledge, there is no published

research on the use of thyme oil in lemon juice marinade with added Yucca extract for inactivating *Salmonella enterica* on raw chicken breast meat. Accordingly, the main objective of the present study was to evaluate the survival of *Salmonella enterica* on artificially inoculated raw chicken breast meat marinated in lemon juice with added thyme oil and Yucca extract. A secondary objective was to determine the extent of sub-lethal injury in *S. enterica* survivors on the marinated chicken breast meat.

Materials and Methods

Bacterial strain and culture conditions

Five serotypes of *Salmonella enterica* (Enteritidis ATCC13076, Heidelberg ATCC 8326, Typhimurium ATCC 14802, Gaminara ATCC 8324, and Oranienburg ATCC 9239) were obtained from the culture collection of the Microbial Food Safety Laboratory at Iowa State University. Those cultures were maintained frozen (-80°C) in brain heart infusion (BHI) broth (Difco; Becton, Dickinson and Company, Sparks, MD) with 10% (v/v) added glycerol. Each frozen stock culture was thawed in cold running water and activated in tryptic soy broth (Difco; Becton, Dickinson and Company, Sparks, MD) supplemented with 0.6% (w/v) yeast extract (TSBYE) at 35°C. Prior to each experiment, two consecutive 24-h transfers of each activated stock culture were performed in TSBYE (35°C) to prepare separate working cultures.

Preparation of inoculum

Equal volumes (6-mL) of each of the five working cultures of *Salmonella enterica* were combined in a sterile centrifuge tube. The cells were harvested by centrifugation (10,000 x g, 10 min, 4^0C) using a Sorvall Super T21 centrifuge (American Laboratory Trading, Inc., East Lyme, CT). The pelleted cells were suspended in 3.0 mL 0.85% (w/v) NaCl (saline) saline to obtain a final viable cell concentration of 10 log10 colony-forming units (CFU)/mL for use in the in-vitro experiments. For inoculation of chicken breast meat samples pelleted cells re-suspended in 30 mL

of saline were to give 9.0 Log10 CFU/mL were used. Colony counts of the cell suspensions were evaluated by serially diluting (10-fold) and surface plating samples on tryptic soy agar (Difco; Becton Dickinson) supplemented with 0.6% yeast extract (TSAYE) followed by counting bacterial colonies on TSAYE after incubation (35^0C) for 24 h.

Minimum inhibitory concentration and minimum bactericidal concentration

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of thyme oil for *S. enterica* were performed using a broth dilution assay (Lopez-Malo et al. 2007) with some modifications. A stock solution of BHI broth containing 0.5% of filter-sterilized Yucca extract was prepared and thoroughly mixed by vortexing. To 99mL of the prepared stock solution, 1mL of filter sterilized thyme oil was added to give a starting concentration of 1.0%. Sterile dilution tubes were labelled as positive and negative controls, the rest were labeled with the following concentrations, 1.0, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.015625%. Thyme oil was serially diluted by first aseptically transferring 10mL of BHI broth containing Yucca extract and 1.0% Thyme oil to the first dilution tube labelled 1.0%. Aseptic transfer of 5.0ml of filter sterilized broth to the rest of the tubes in the order listed above. Viable cell concentrations of *S. enterica* were diluted to obtain a final of log 5.0 CFU/ml. The tubes were then inoculated and incubated at 35^0C for 24 hours. The tubes were checked for turbidity after 24 hours.

To determine the Minimum Bactericidal Concentration (MBC) for thyme oil, 0.1mL aliquots from the tubes showing no turbidity were surface plated on TSAYE for 48 hours at 35^0C. The lowest concentration of thyme oil that demonstrated a 3-log kill was considered as the MBC.

Preparation of Lemon Juice marinade

Whole Sunkist® lemons from the same production lot were purchased from a local grocery store in Ames, Iowa. The lemon juice for the marinade solutions was prepared by juicing the lemons using a manual citrus juicer using a commercial home juicer (Hamilton Beach (67601A, Electric, 800 Watt) in sterile beakers. Prior to each experiment four treatment solutions including control (lemon juice alone) were prepared by aseptically transferring 100mL of lemon juice into each of four sterile screw capped glass bottles. A filter-sterilized stock solution of yucca extract (Garuda International, Inc, Exeter, CA) in lemon juice was added to one bottle to obtain a final yucca extract concentration of 0.5% (w/v). To two of the bottles a filter-sterilized stock solution of certified food grade thyme oil (Sigma-Aldrich, Milwaukee, WI) emulsified in yucca extract in lemon juice was added to give final thyme oil concentrations of 1µL/mL and 2µL/mL, respectively, with each marinade solution having the same concentration of yucca extract [0.5% (w/v)]. The bottles containing the different marinade solutions were capped, vigorously shaken and stored at 4^0C until used.

Inoculation of lemon juice marinade treatment solutions

For in vitro time-kill studies, 20mL aliquots of each of the four marinade solutions were aseptically transferred to sterile 50-mL plastic tubes and inoculated by addition of 0.2 mL of a five-strain cocktail of *Salmonella enterica* to obtain a final viable cell concentration of 8.0 $\log_{10}$ colony-forming units (CFU)/mL. Each tube of inoculated treatment solution was thoroughly mixed by vortexing and samples were removed for microbial analysis at 0, 3, 6, 9, 12 and 15 minutes. Time 0 min actually represents about 15 seconds of exposure of the pathogen to the marinade before transferring samples of inoculated marinade to BPW for further dilution and plating.

Preparation and inoculation of chicken breast meat

Fresh, skinless chicken breast fillets were obtained from a local grocery store and transported on ice in a cooler to the Microbial Food Safety Laboratory at Iowa State University. The fillets were refrigerated at 4^0C and used within 24 hours of purchase. Five chicken breast fillets were randomly selected and samples (50-g each) of chicken breast meat were aseptically excised using a sanitized cylindrical plastic corer 30mm in diameter. Each sample was inoculated with 0.1mL of a five-strain cocktail of *Salmonella* (~9.0 log CFU/mL) to obtain a final concentration of ~8.0 Log_{10}CFU/sample. The inoculum was spread over the surface of the meat sample using a sterile bent glass rod. The inoculated chicken breast slices were then held for 30 minutes at 23^0C in a laminar flow bio-hazard chamber (with the fan on) and for an additional 1.5 h to allow the inoculum to dry.

Marination of inoculated chicken breast meat

For marination, four 50-g samples of inoculated chicken breast slices were each transferred to a separate sterile beaker containing a marinade treatment solution at ambient temperature (22 ± 1 C). Each chicken breast meat sample was immersed (inoculum side down) in the marinade solution. The ratio of marinade to meat was 1:2 (50g/100mL). At set time intervals meat samples were removed from the marinade, drained for 30 seconds on a sanitized stainless-steel grill and then analyzed for *Salmonella* survivors.

Microbiological analysis

For the in-vitro study, microbiological analysis of the inoculated marinade treatment solutions was carried out after 0, 3, 6, 9, 12 and 15 minutes. Ten-fold serial dilutions of each treatment solution were prepared using sterile double strength BPW (pH 7.2). Aliquots (0.1-mL) of diluted treatment solutions were surface plated (in duplicate) on XLT-4 agar plates. The inoculated agar plates were incubated at 35^0 C and bacterial colonies were counted after 48 h.

For determination of *Salmonella* survivors in the inoculated chicken breast meat held in different marinade solutions, the samples were taken from each treatment at 0 (control), 2, 4, 6 and 8 hours. The samples were drained for 30 seconds on a sanitized stainless-steel grill. The samples were then transferred to stomacher bags each containing 50mL of double strength BPW and pummeled for 1.0 min in a laboratory stomacher blender operating at medium speed. Aliquots (1-mL) of the sample homogenate were serially diluted in BPW and 0.1-mL portions were spread-plated on both XLT-4 agar plates and XLT-4 plates overlaid with TSAYE (TAL). The inoculated agar plates were then incubated 35^0 C and bacterial colonies were counted at 48 h.

Determination of sub-lethal injury

For the determination of sub-lethally injured cells, a recovery method, the thin agar layer (TAL) method, developed by Kang and Fung in 1999 was used with modifications. Briefly, preparation of the TAL medium involved aseptically overlaying 15mL of sterile TSAYE (49^0C) onto 25mL of solid XLT agar in petri dishes. Sub-lethal injury in the surviving microbial population was determined using the method by Wuytack et al. (2003). Using survivor curves based on recovery of microbial colonies on both selective and modified selective media (XLT-4 overlaid with TSAYE), injury was expressed as a logarithm of the reduction factor (RF), which is the ratio of colony counts (CFU/ml) of the control to the counts (CFU/ml) of the treated sample (Fei et al. 2018).

$$Log\ RF = Log\left(\frac{CFU\ before\ treatment(control)}{CFU\ after\ treatment}\right)$$

The log RF was calculated from bacterial colony counts recovered from XLT-4 and were plotted on the Y-axis against colony counts recovered from XLT-4 overlaid with TSAYE on the X-axis. Linear regression lines were fitted through data points. The extent of sub-lethal injury was

determined by comparing the slopes and intercepts of the regression lines for each treatment at a 5% statistical significance level.

Thyme Oil GCMS Analysis

The thyme essential oil sample was analyzed with Agilent Technologies Model 6890A Gas Chromatography system coupled to a Model 5973N inert XLMSD with Triple –Axis Detector. Capillary column Agilent Rxi-5SilMS (30m x 0.25mm x 0.25mm) was used. Each sample injected consisted of 1µL of essential oil diluted in 1 ml Hexane HPLC grade using split-less injection. The inlet temperature was 250°C and the helium flow rate was 1.0 mL/min. Ionization voltage was 70 eV with interface temperature of 280°C. The MS source temperature was 230°C and MS Quad temperature was 150°C. The sequence temperature was: initial temperature 50°C, ramp 5°C per minute to 180°C, then 10°C per minute to 280°C with a total run time of 36 min per sample. A mixture of homologous series of normal alkanes from C10 to C26 was analyzed under the same conditions as listed above.

The compounds present in the essential oil were identified by comparing mass spectra of each component with those from NIST by AMDIS (Automated Mass Spectral Deconvolution and Identification System). The identification was also based on the comparison between the literature and estimated Kovat′s retention indices using the formula:

$RI_x = 100\ [n + (t_x\text{-}t_n) / (t_{n+1} - t_n)]$ (Van den Dool and Kratz 1963)

t_n and t_{n+1} represent retention times of the reference normal alkane hydrocarbons eluting closely before and after the chemical compound to identify “x”, t_x is the retention time of that compound “x” and “n” represents the number of carbons.

Statistical analysis

All experiments were replicated three times and results are reported as averages. SAS software (SAS version 9.3, SAS Institute, Cary, N.C.) was used for performing two-way Analysis of Variance (ANOVA) to evaluate treatment means with significant differences. The Welch test was used to determine significant differences between paired treatments. Tests were carried out at a 5% significance level.

Results and Discussion

Results

Minimum inhibitory concentration and minimum bactericidal concentration

Thyme oil concentrations ranging from 1.0% to 0.03% completely inhibited growth of *Salmonella enterica* in BHI broth (pH 7.4). The MIC of thyme oil for the pathogen in BHI broth held at 35^0C for 24 hours was 0.03%. The MBC of thyme oil was 0.06%, which resulted in 3.2 log CFU/mL reduction in initial viable count of *S. enterica*. The initial count of 5.0 $\log_{10}$ CFU/mL was reduced to 1.8 $\log_{10}$ CFU/mL.

Background microflora and pH of marinade solutions

Microbial analysis of the lemon juice showed low counts of background flora (~1.8 $\log_{10}$ CFU/mL) based on bacterial colony counts on TSAYE. The initial pH of the lemon juice was 2.34 at 25^0C. There were no significant changes in the pH of the marinade solutions after addition of Yucca extract and or thyme oil (data not shown).

Viability of pathogens in marinade solutions

Figures 1A and 1B show numbers of *S. enterica* survivors in lemon-based marinade solutions with or without added yucca extract or yucca extract combined thyme oil (0.1% or 0.2%). Those figures present colony counts of the pathogen on TAL plates (Figure 1A) and XLT-4 agar plates (Figure 1B). The initial viable count of *S. enterica* in artificially inoculated lemon juice and

lemon juice with added Yucca extract and thyme oil was 8.0 ± (0.4) $\log_{10}$ CFU/mL. All treatments decreased initial numbers of survivors with lemon juice containing yucca extract combined with TO (0.2%) exhibiting the highest antibacterial effects. Lemon juice alone decreased numbers of the pathogen from 8.21 to 5.96 log CFU/mL after 15 minutes (Figure 1A). Survivors in lemon juice with added yucca extract alone were consistently lower than those in lemon juice alone; however, differences were not statistically significant ($p>0.05$). Both treatment solutions with added yucca extract and TO (0.1% and 0.2%) exhibited the strongest bactericidal effect against the pathogen (Figures 1A and 1B). While there were *S. enterica* survivors in all other treatment solutions after 15 minutes, the pathogen was not detected in lemon juice with added yucca extract and TO (0.2%) after 12 minutes (Figure 1A). In all instances from 3 to 15 minutes lower survivors of *S. enterica* colonies were observed on XLT-4 agar plates compared to TAL plates ($P<0.05$).

Survival of pathogens inoculated on chicken breast fillets

Figures 2A and 2B present the numbers of *S. enterica* survivors on raw chicken breast meat marinated in lemon-based treatment solutions at 22 ± 1 ^{o}C. The initial viable count of *S. enterica* artificially inoculated on chicken breast fillets was 8.0 ± (0.4) $\log_{10}$ CFU/sample based microbial analysis of the cell suspension used to inoculate the chicken samples. The populations of survivors on the inoculated chicken breast fillets after they were held for 2 hours at ambient temperature (22 ± 1 ^{o}C) were 7.98 log CFU/sample. All treatments with added yucca extract and TO significantly reduced initial numbers of viable *S. enterica* on raw chicken meat samples compared to control and lemon juice with added yucca extract alone ($P<0.05$). Based on initial populations of viable S. enterica on chicken samples in lemon juice (control) and lemon juice with yucca extract alone decreased by 2.25 Log and 2.46 Log, respectively, in 8 hours. In contrast, initial populations of the pathogen on chicken immersed in lemon juice with added yucca extract and TO decreased by

3.58 Log (0.5% TO) and 4.81 Log (1.0% TO) after 8 hours. There was a significant difference in the number of survivors plated on both TAL and XLT media ($P<0.05$).

Sub-lethal injury of Salmonella enterica

Table 1 shows sub-lethal injury (expressed by linear regression parameters) in *S. enterica* survivors resulting from exposure of the pathogen (artificially inoculated chicken breast meat) to the marinades. That table shows the slopes and intercepts from linear regression plots showing reduction in culturability. Those data represent an index of sub-lethal injury in the surviving population of *S. enterica* inoculated on chicken breast meat. Diluted homogenates of marinated chicken were plated on selective and modified selective media following marination in lemon-based marinade with or without added thyme oil. To calculate the log Reduction Factor, bacterial counts (CFU/ml) from XLT, a selective agar, were plotted against bacterial counts (CFU/ml) from XLT4 overlaid with TSAYE (TAL), a modified selective media. Linear regression lines were fitted through the data points (Wang et al. 2018). Slopes and intercepts from each plot representing a treatment replication were obtained and compared with each other. The extent of injury inflicted on a microbial population was compared using slopes and intercepts (Table 1).

The linear regression plots generated slopes and intercepts for each treatment. To observe sub-lethal injury, we had to determine the level of significance in which the slope deviated from 1.0. When the counts from XLT4 overlaid with TSAYE (TAL) are equal to the counts on XLT4 alone, there is no sub-lethal injury observed and therefore the slope is 1.0. However, when the counts on XLT overlaid with TSAYE (TAL) are greater than those observed in XLT alone, we can deduce that sub-lethal injury has occurred, and the slope is greater than 1.0. When the intercept is 0, there is no injury observed because the counts on the two types of media are the same. A significant positive deviation i.e. intercepts >0 is an indicator of sub-lethal injury.

Based on results of statistical analysis cells of *Salmonella enterica* exhibited no significant sub-lethal injury across the four treatments (P>0.05). Based on bacterial populations we observed that longer exposure of the cells to marinade solutions resulted in larger differences in populations obtained from selective and non-selective media indicating sub-lethal injury in the surviving populations of the pathogen. However, those differences were not statistically significant (P>0.05). Based on the slope and intercept parameters there was no significant sub-lethal injury in the populations of survivors (P > 0.05).

GCSM analysis of thyme oil

Table 2 shows the compositional analysis of thyme oil (TO) used in the present study. Fifteen different components were identified representing major and minor components of that essential oil. The concentrations of components ranged from 0.14% to 51.07%. Thymol and O-cymene were the top two major components at concentrations of 51.07% and 24.1%, respectively.

Discussion

Numerous peer-reviewed and published studies have shown the potential of plant-based essential oils such as thyme oil to control foodborne pathogens since they possess both bactericidal and bacteriostatic attributes (Thanissery and Smith, 2014; Van Haute *et al.*,2016; Manu *et al.*, 2017) which is consistent with our findings. Yang *et al.,* 2013 reported that marinating beef in lemon juice inactivated *S. enterica* inferring that lemon juice intrinsically has antibacterial properties. Although the inhibitory effect of lemon juice against pathogenic bacteria is well known, results of the present study indicate that *S. enterica* can survive for 8 hours (and possibly more) on chicken breast meat immersed in lemon juice (Figure 2).

We investigated the survival and sub-lethal injury of *S. enterica* in lemon-based marinade containing Yucca extract as a natural surfactant and thyme oil as an antimicrobial agent. Our

decision to use yucca extract is mainly because of the immiscibility of essential oils in highly polar or hydrophilic solutions such as lemon juice justified by studies conducted by Samperio *et al.,* 2010. Our study also demonstrated that the use of only lemon juice to marinate meat might not completely inactivate *S. enterica* thus consumers are potentially at risk of contracting a foodborne illness further justifying the need of thyme oil.

The viability of *S. enterica* in marinade solutions showed a rapid decline in microbial counts after 15 minutes in both controls and treatments containing 0.1% and 0.2% thyme oil. The low pH (2.34) of the lemon juice and the citric acid found in lemon juice are likely responsible for the antimicrobial action of lemon juice, as similar findings were reported by others (Bjornsdottir et al., 2006; J. Yang *et al.,* 2013). The addition of Yucca extract to the lemon juice resulted in a better kill of *S. enterica*; however, the results were not statistically significant from those of lemon juice alone. This might be due to the presence of saponins in the yucca extract as saponins are known to have antimicrobial properties (Avato. P *et al*., 2006). The addition of thyme oil to the lemon juice with added Yucca extract further exhibited a stronger bactericidal action, which was a statistically significant effect compared to the controls without thyme oil (Boscovic *et al.,* 2015; Possas *et al.,* 2017).

There were more *S. enterica* survivors on the artificially inoculated chicken breast fillets compared to numbers of survivors of the pathogen in marinade solutions. This is partly attributed to the protective effect that the chicken breast fillet offers *S. enterica* upon attachment. There is a possibility that during attachment, there is formation of biofilm that reduces the contact of the pathogens with the marinade treatment solutions; however, this is not possible when the salmonellae are as planktonic cells directly exposed to marinade solutions. Yang *et al.,* 2016 and Dimakopoulou-Papazoglou *et al.,* 2016 reported an increased tendency of *S. enterica* to produce

biofilm rapidly especially when they were exposed to conditions of very low pH. Also, an increase in pH of the marinades was observed over the 8-hour period probably because of the buffering effect of protein in meat juices from the chicken breast fillets. As protein approaches its isoelectric point upon denaturation by acid, meat exudate increases and causes an increase in pH of the marinade solution. This in turn can potentially allow increased survival of *S. enterica* in the marinade solutions.

With regard to sub-lethal injury in surviving populations of *S. enterica*, the low pH (2.4) of lemon juice and bactericidal properties of thyme oil are probably responsible for the injury observed during the study. Sub-lethally injured cells were unable to form colonies on selective media (XLT4) but were able to repair their injury on the TSAYE that overlaid the selective agar (XLT4) and eventually form colonies. From a food safety perspective, our observations bear serious implications for the food industry because the counts on the selective media were always lower than those observed on selective agar overlaid with non-selective agar. This result suggests that use of selective agar for microbial analyses in the food industry can under-estimate the actual viable counts of *Salmonella* present in the food thus presenting a significant food safety risk. More importantly sub-lethally injured cells can repair their lesions and subsequently multiply to infectious doses to cause a foodborne disease outbreak or a product recall. Our findings bear significant biological implications given that some foodborne pathogens have very low infectious doses that might be overlooked if they are sub-lethally injured and thus cannot be detected in selective media.

Sub-lethal injury was observed in surviving populations of *S. enterica* after inoculation in marinade solutions and artificially on chicken breast fillets. Wesche *et al.*, 2009 reported that sub-lethal injury of cells occurred because of continuous exposure to chemical or physical agents that

critically injure microorganisms but not lethally. Physiologically, these cells are unable to replicate when plated and incubated in selective media alone. In our experiment, we used the TAL method developed by Kang and Fung, 1999 but with slight modifications for resuscitation and recovery of sub lethally injured cells. There was a significantly high population of injured cells among the surviving population that was inoculated directly into marinade solutions compared to the cells that were inoculated directly onto the chicken breast fillets. These results provide evidence that conventional selective media might be underreporting the number of surviving populations of foodborne pathogens in food putting consumers at risk of contracting foodborne illnesses. The additional layer of a non-selective media such as TSAYE allow the microorganisms to repair their injuries, resuscitate and replicate. The ability of injured cells to resuscitate makes them important in food pathogen control as they might go undetected during routine quality control practices in industry (Johnson and Busta, 1984; Wesche *et al.*, 2009).

The minimum inhibitory concentration of thyme oil was low (0.03125%). This was the lowest concentration that was required to inhibit the growth of *S. enterica* in BHI broth incubated overnight. MBC is the lowest amount of thyme oil that resulted in at least 3 Log_{10} reduction of *S. enterica* in broth. Lambert R.J *et al.*, 2001 reported that the determination of MIC of any EO such as thyme oil especially during investigative studies determining the use of an EO as an alternative to preserve or extend the shelf life of food was vital. This is because high concentrations of EOs may confer unfavorable organoleptic properties to food that may be undesirable to consumers. Determination of MIC and MBC helps researchers find the balance between what doses to apply in food to kill pathogens but not alter organoleptic properties of food significantly.

The GCMS results of thyme oil revealed that this EO contained thymol which was highest in concentration (51.07%) followed by O-cymene (24.1%). Lambert R.J *et al.*, 2001 proposed the

mechanism of action of thymol as having the potential to damage membrane integrity of cells, resulting to ion leakage that ultimately compromises cytoplasmic pH of the cell leading to death of the cell. The other numerous components making up thyme oil also further contribute to the bactericidal properties of thyme oil as these components may be acting synergistically against foodborne pathogens further explaining their potential to inhibit growth of diverse microorganisms.

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Table 1 Slopes and intercepts from plots showing reduction in viability of *Salmonella enterica* on chicken breast meat in lemon-based marinade.

Treatment	Slope	Intercept	R-Squared
Lemon juice alone	1.283 ± 0.227	0.349 ± 0.227	0.918
Lemon+ Yucca	1.223 ± 0.066	0.104 ± 0.047	0.857
Lemon+ Yucca+ 0.5% Thyme oil	1.235 ± 0.245	-0.222 ± 0.736	0.907
Lemon+ Yucca+ 1.0% Thyme oil	1.204 ± 0.108	0.431 ± 0.291	0.889

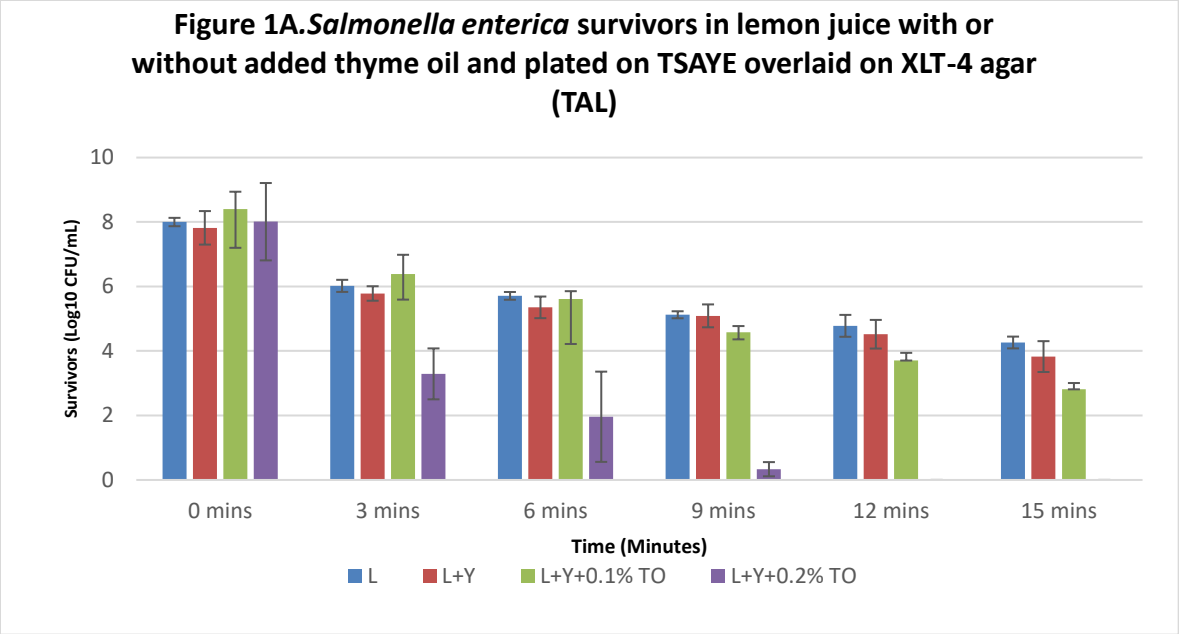
[a] Value for slope is significantly different from 1 ($P<0.05$)

Table 2: Compounds identified in thyme essential oil by GCMS analysis

Compouds	RT	RI_s	RI_{Nist}	Percent (%)
α-pinene	5.98	942.5	937	1.78
Camphene	6.37	956.8	952	0.42
β-Myrcene	7.29	990	991	0.55
o-Cymene	8.24	1024.3	1022	24.1
Eucalyptol	8.45	1032	1032	1.67
γ-Terpinene	9.14	1057.2	1060	5.42
4-Carene/Linalool	10.27	1097.9	1099	4.40
Camphor	11.63	1147	1145	1.49
Isoborneol	12.09	1164	1157	0.14
Endo-borneol	12.32	1172.6	1167	0.73
Terpinen-4-ol	12.56	1181.2	1177	0.72
Thymol	15.57	1293.2	1291	51.07
Carvacrol	15.82	1301.5	1299	3.33
Caryophyllene	18.99	1422.6	1419	0.20
Caryophyllene oxide	22.95	1585.4	1581	0.18

RT: Retention time, RI_s: Kovat′s Retention index of thyme oil compounds found in the sample used in this study; RI_{Nist}: Kovat′s Retention index in NIST14 library.

A



B

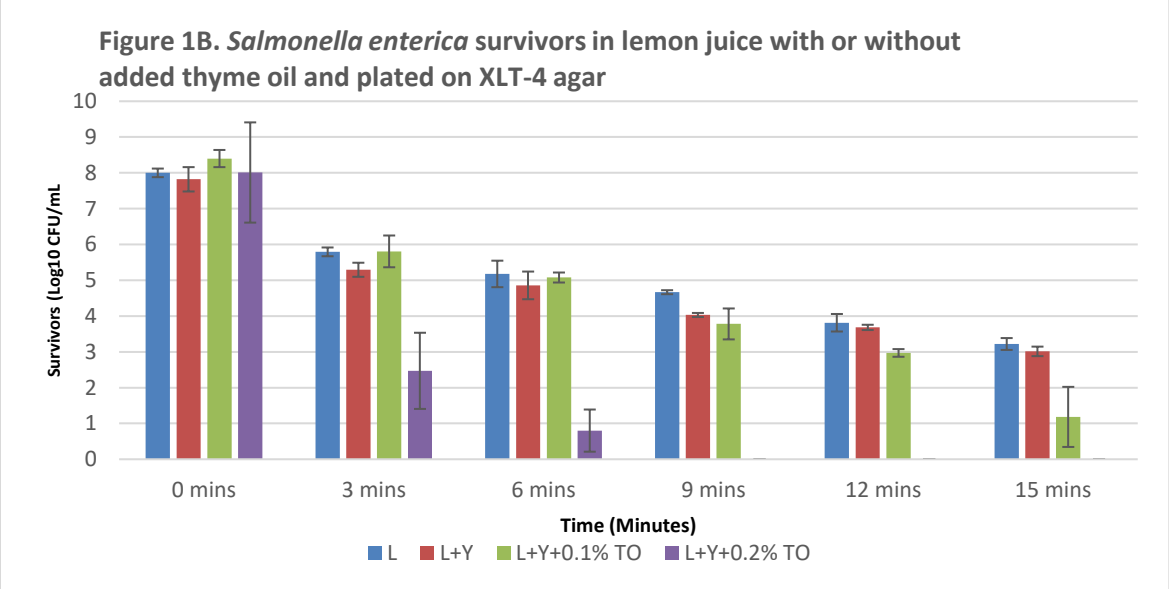
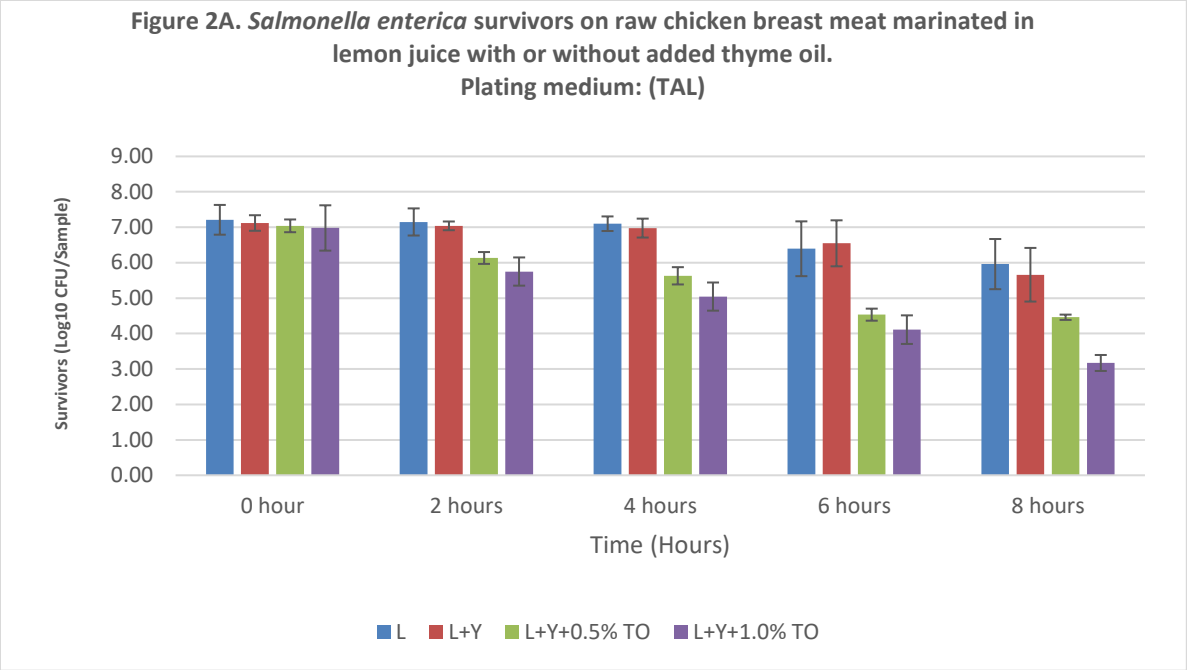


Figure 1. *Salmonella enterica* survivors in lemon juice marinade alone with or without added thyme oil and Yucca extract

*Numbers of survivors shown in A and B are derived from colony counts on TSAYE (thin-layered on XLT-4) and on XLT-4, respectively



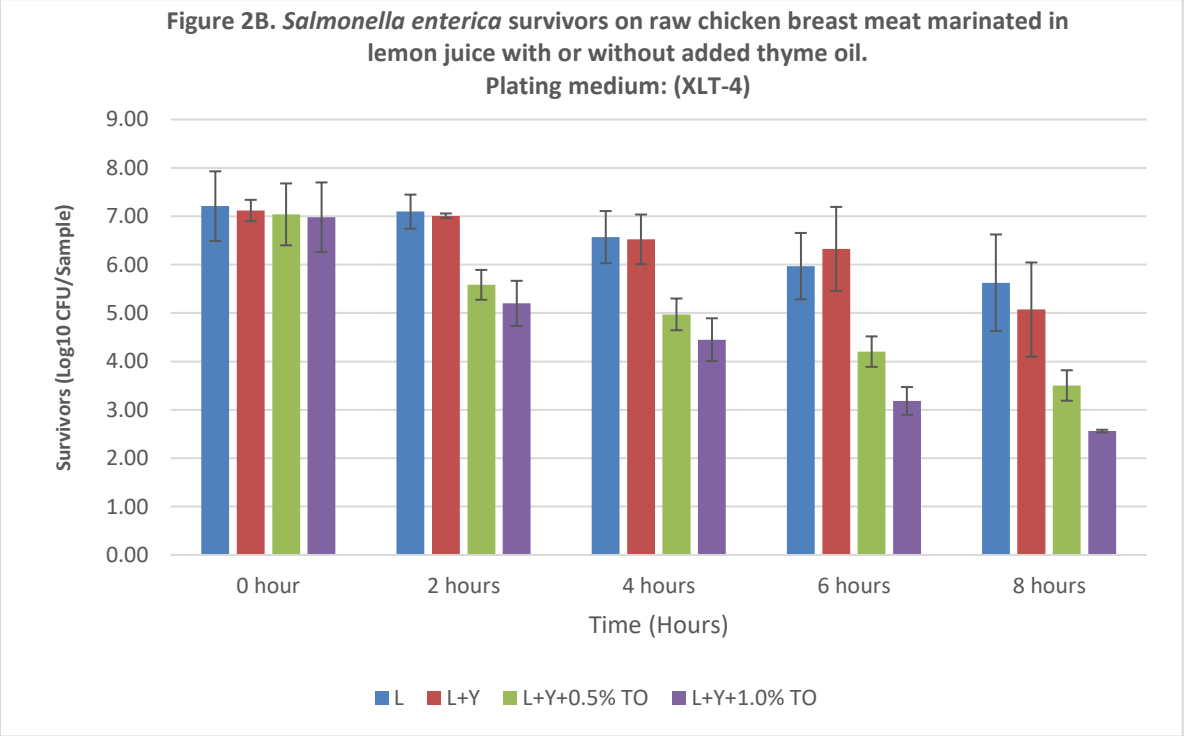


Figure 2. *Salmonella enterica* survivors on raw chicken breast meat marinated in lemon-juice with or without added thyme oil.

CHAPTER 4. *SALMONELLA* TYPHIMURIUM AND *LISTERIA MONOCYTOGENES* IN THE LONG-TERM-SURVIVAL PHASE EXHIBIT INCREASED TOLERANCE TO CINNAMALDEHYDE IN 0.85% SALINE AND IN APPLE JUICE

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Abstract

The heightened tolerance of long-term survival phase (LTS) cells of pathogens to antimicrobial agents can pose a substantial threat to food safety. The objective of this study was to investigate the tolerance of LTS cells of *Salmonella* Typhimurium ATCC 14028 and *Listeria monocytogenes* Scott A to cinnamaldehyde in 0.85% (w/v) saline and in apple juice. The *S.* Typhimurium and *L. monocytogenes* were cultured in tryptic soy broth supplemented with 0.6% (w/v) yeast extract (TSBYE; 35 ^{o}C) for 24h to obtain stationary phase (STAT) cells and for 14 days in TSBYE (35 ^{o}C) to obtain LTS cells. The LTS (~10^5 CFU/mL) and STAT (~10^8 CFU/mL) cells of both pathogens were exposed to 0.1% and 0.05% cinnamaldehyde in 0.85% saline and in apple juice, respectively, for 5, 10, 15 and 20 minutes. Saline or apple juice without added cinnamaldehyde served as control. The STAT and LTS survivors of both pathogens were plated on xylose-lysine-tergitol 4 agar (XLT4) and Modified Oxford agar (MOX) and on tryptic soy agar with 0.6% yeast extract (TSAYE) at and enumerated after 48h of incubation (35 ^{o}C). After each exposure of the pathogens to cinnamaldehyde in saline (pH 5.7) and apple juice (pH 3.62), a greater number of LTS cells survived compared to STAT cells ($P<0.05$). The LTS cells exhibited higher D-values compared STAT cells in both saline and apple juice ($P<0.05$). In apple juice, the D-values for STAT cells of *S.* Typhimurium and *L. monocytogenes* were 4.47 and 3.89, respectively. In contrast, D-values of LTS cells were 6.47 (*S.* Typhimurium) and 5.48 (*L. monocytogenes*). The extent of sub-lethal injury in surviving STAT cells was significantly greater compared to LTS survivors ($P<0.05$). Based on these results, LTS cells of *S.* Typhimurium and *L. monocytogenes* are far more tolerant to cinnamaldehyde compared to STAT cells and should be considered as the target organisms in the development of antimicrobial processes involving cinnamaldehyde.

Introduction

The demand for minimally processed foods is on the rise as consumers express strong preferences for foods devoid of synthetic chemical preservatives (Hyldgaard *et al*., 2012; Calo *et al*., 2015, Wang *et al*., 2018; Thomas-Popo *et al*., 2019). Traditionally, the food industry largely controls spoilage and pathogenic bacteria by employing synthetic chemical preservatives such as benzoates or sorbates to kill undesirable microorganisms in food (Calo et al., 2015, Thomas-Popo et al., 2019). However, the use of synthetic chemicals has generated a negative perception among consumers as some studies have associated prolonged exposure to some chemical preservatives with development of certain cancers, teratogenicity or acute toxicity (Faleiro, 2011). The demand for more natural food products has prompted the food industry to explore new and alternative methods of food preservation. In this respect, plant essential oils (EOs) have gained much attention as potential natural preservatives for foods (Pandey et al., 2017; Mendonca et al., 2018)

The EOs and their components have become popular alternatives to synthetic preservatives because they have exhibited tremendous antimicrobial properties and could be used to achieve food safety (Bajpai *et al*., 2011; Manu *et al*., 2017; Pandey et al., 2017). The application of EOs in fruit juices is an example of a non-thermal approach to enhance the safety and microbiological quality of juices without damaging heat labile nutrients such as ascorbic acid while preventing or minimizing negative organoleptic changes that may occur in heat-processed juices (Manu *et al.*, 2017; Mendonca *et al*., 2018; Thomas-Popo *et al*., 2019). There is a vast number of peer-reviewed reports that show the efficacy of plant EOs or EO components in the inactivation of pathogenic and spoilage bacteria in fruit juices.

Cinnamaldehyde is a component of cinnamon oil that is Generally Recognized as Safe (GRAS) under code of federal regulations (USDA 21 CFR 182.60) for use as a flavorant in foods such as flavored drinks and juices. There are several published reports that show cinnamaldehyde

has a great potential in activating undesirable microorganisms in food. Jo et al. (2015) demonstrated that encapsulated cinnamaldehyde in a nano-emulsion significantly reduced populations of *Salmonella* Typhimurium *and Staphylococcus aureus* in water melon juice. Friedman (2017), reported that cinnamaldehyde exhibited antimicrobial activity against *Bacillus cereus, Campylobacter jejuni, Clostridium perfringens, Escherichia coli, Listeria monocytogenes,* and *Salmonella enterica* during in *vitro* laboratory experiments and in animal feed. Manu et al. (2017) also reported a significant decline (5 log reduction) in populations of *Escherichia coli* O157:H7 and *Salmonella enterica* when those pathogens were exposed to cinnamaldehyde (1.5 or 2.0 µL/mL) in a mixed berry juice (1.5 µL/mL) and carrot juice (2.0 µL/mL) held at 4 or 12^0 C.

Enteric pathogens such as *Salmonella spp* and *Listeria monocytogenes* can survive for prolonged periods of time in food processing environments and equipment to cause recurrent contamination of food and pose a significant food safety threat (Harvey & Gilmour, 2001; Møretrø, & Langsrud, 2004; Ratani *et al*., 2012; Ferreira *et al*., 2014) to result in foodborne disease (Scallan *et al*., 2011). The prolonged survival of these pathogens is likely associated with a life cycle phase called the long-term survival (LTS) phase, which is described as a cellular state whereby viable populations of microorganisms remain dormant for a long period of time, months or years (Kolter *et al*., 1993; Wen et al., 2009; Wang et al., 2018). Conventionally, the microbial life cycle has been characterized by four phases namely, lag, exponential, stationary and death phase (Kolter *et al.*, 1993). However, the LTS phase is recognized as the fifth phase in the bacterial life cycle where the cells are considered dormant (Finkel, 2006). Nonetheless, the existence of pathogenic bacteria such as *L. monocytogenes* and *S.* Typhimurium in the LTS phase has significant implications to food safety because those pathogens persist long enough in the natural environment and in food processing facilities to cause high incidences of recurrent contamination of foods.

For decades, stationary phase cells have been used in the design and validation of antimicrobial processes in the food industry (Wang et al., 2018). The utilization of stationary phase cells in microbial challenge studies has become scientific dogma because they are believed to be more hardy that exponential phase cells. Also, the use of stationary phase cells in microbial food safety research has continued over decades although LTS phase cells have long been characterized since in the 1930s (Steinhaus and Birkeland, 1939). The justifiable use of stationary phase cells in pathogen challenge studies is questionable because enteric pathogens that contaminate our foods invariable come from environments that induce their entry into the LTS phase. This is in contrast to the stationary phase attained by bacteria incubated overnight in nutrient-rich laboratory media at optimal growth temperature, water activity, pH and lack of microbial competitors (Finkel, 2006; Navarro Llorens *et al.*, 2010; Wang et al., 2018).

There is a growing number of reports that link LTS phase cells of pathogens to increased tolerance to both physical and chemical antimicrobial agents. For example, Wen et al. (2009) showed that LTS phase cells of *L. monocytogenes* were more tolerant and survived better compared to stationary and exponential phase cells of the same pathogen when they were exposed heat or high pressure. Wang et al. (2018) demonstrated that LTS cells of *S.* Typhimurium were more resistant to UV radiation compared to stationary and exponential cells in apple juice or saline (0.85% NaCl). More recently the LTS cells of *S.* Typhimurium were shown to be more persistent and tolerant to disinfectants compared to stationary and exponential cells when inoculated onto surfaces (Djebbi-Simmons et al., 2019).

There is an increasing number of published reports on the relatively high tolerance LTS cells of pathogens to certain physical and chemical antimicrobial interventions. Considering the growing interest in use of EOs and EO components as natural antimicrobials in foods, the killing

effects of those antimicrobials against LTS phase pathogens warrant investigation. Accordingly, the main objective of the present study was to investigate the tolerance of LTS phase *S.* Typhimurium and *L. monocytogenes* to cinnamaldehyde in artificially inoculated apple juice. A secondary objective was to evaluate the extent of sub-lethal injury in survivors of stationary and LTS phase cells following their exposure to cinnamaldehyde in 0.85% NaCl (saline) and in apple juice.

Materials and Methods

Bacterial strain and cultural conditions

The cultures used in this study were *L. monocytogenes* Scott A and *S. enterica* serovar Typhimurium ATCC 14028 and were obtained from the Microbial Food Safety Laboratory culture collection at Iowa State University. The stock cultures were stored frozen (-80^0 C) in brain-heart infusion (BHI) broth (Difco; Becton, Dickinson and Company, Sparks, MD) with added 10% (v/v) glycerol. The frozen stock cultures were thawed and resuscitated in Tryptic Soy Broth with added 0.6% Yeast Extract (TSBYE) incubated at 35^0C. To prepare working cultures prior to each experiment, two-consecutive 24-hour transfers of the thawed stock cultures were performed by incubating the cultures in TSBYE (35^0C).

Preparation of Stationary phase and LTS cells

To prepare the stationary phase (STAT) cells and LTS cells for *S.* Typhimurium and *L. monocytogenes*, 1-mL aliquots from the working cultures of both organisms were each aseptically transferred to separate 100-mL portions of TSBYE in screw-capped 250mL Erlenmeyer flasks. Upon inoculation, the flasks of TSBYE were slightly swirled to allow for proper dispersion of inoculum and were then incubated at 35^0C. The STAT cells were harvested after 18 hours and LTS cells after 14 days.

Determination of cell viability

For determination of cell viability, harvested STAT and LTS cells for both *S.* Typhimurium and *L. monocytogenes* were serially diluted (10-fold) in 0.1% peptone (Difco) and 0.1mL aliquots from both physiological states of the two pathogens were surface plated on tryptic soy agar (TSA; Difco) supplemented with 0.6% yeast extract (TSAYE) and on xylose-lysine-tergitol 4 (XLT-4) agar (Difco) for *S.* Typhimurium and on TSAYE and modified oxford agar (MOX) for enumeration of *L. monocytogenes.* The agar plates were then incubated aerobically at 35^0 C and bacterial colonies were counted after 48h.

MIC and MBC

The minimum inhibitory concentration (MIC) for stationary phase cells for both *S.* Typhimurium and *L. monocytogenes* was carried out in BHI (pH 7.2 ± 0.2) while the minimum bactericidal concentration (MBC) for the LTS cells for the two pathogens was carried out in saline (0.85% [w/v]; pH 5.7 ± 0.1). For each experiment, all test solutions were at ambient temperature (22 ±1 ^{o}C). The MIC and MBC assays in this study were carried out using a broth dilution assay adopted from Lopez-Malo et al. (2007). For the MIC of STAT cells of *S*. Typhimurium and *L. monocytogenes*, a 0.5-mL aliquot of cinnamaldehyde was sterilized by filtration using a 0.02µm pore size Luer Lok filter and a small Luer Lok syringe and used for preparing doubling dilutions in BHI and saline. Tubes of BHI were labeled as positive (inoculated) and negative (non-inoculated) controls and other tubes of BHI were appropriately labeled with the concentrations of cinnamaldehyde ranging from 10 to 0.15 µL/mL and from 14.4 to 0.11 µL/mL. Doubling dilutions of the highest concentrations of cinnamaldehyde (10 and 14.4 µL/mL) were performed to achieve the desired concentrations of cinnamaldehyde in BHI for the MIC assay. For the MBC assay, the same procedure as previously described for MIC assay for the STAT cells of the two pathogens was repeated except that 0.85% saline was used for preparing doubling dilutions of the

cinnamaldehyde. The STAT and LTS cells were appropriately diluted in 0.85% saline and used to inoculate the tubes of media for the MIC and MBC assays to obtain a concentration of ~ 5.0 Log CFU/ml in each tube.

For determination of MIC, the tubes were checked for turbidity after incubation at 35^0C for 24h and the cinnamaldehyde concentration in the first tube that did not show turbidity from was identified as the MIC. For MBC of STAT and LTS cells, the inoculated saline solutions containing cinnamaldehyde were serially diluted (10-fold) after 24h and lowest concentration of cinnamaldehyde that yielded 3-Log CFU/ml reduction was the MBC.

Preparation and inoculation of saline and apple juice

Commercially available apple juice from the same production lot was purchased from a local store in Ames, Iowa. That juice (pH 3.64) had no added preservatives and was clarified and pasteurized. At our laboratory, the apple juice was sterilized by filtration using a bottle-top vacuum filtration system with 0.22 µm pore size filter (Corning, Amsterdam, Netherlands) and refrigerated at 4^0C until used in experiments. A 0.85% (w/v) NaCl (saline; pH 5.7 ± 0.1) was prepared and sterilized by autoclaving. To prepare treatment solutions, 20µL of cinnamaldehyde was dispersed in 20mL of saline to give a 1 µL/mL (0.1%) concentration cinnamaldehyde in a 50mL pyrex tube and thoroughly mixed by vortexing for 30 sec. For the apple juice, 10µL was added to 20mL of apple juice (pH 3.64; Brix 11.7) to obtain a 0.5 µL/mL (0.05%) cinnamaldehyde concentration and mixed by vortexing as previously stated. The saline and apple juice used in these experiments were tempered to 22 ± 1 ^{o}C prior to inoculation.

Stationary phase and LTS cells, each in 30mL of TSBYE, were harvested by centrifugation (10,000 x g, 10 min, 22 ^{0}C) using a Sorvall Super T21 ultracentrifuge (Sorvall Product, L.P., Newtown, CT). The pelleted cells were then suspended in 0.85% (w/v) and thoroughly mixed by

vortexing. For the in vitro experiment involving saline, 0.2mL of STAT or LTS cells for both *S.* Typhimurium and *L. monocytogenes* and were inoculated in the saline with 0.1% cinnamaldehyde to obtain a final concentration of ~10^8 CFU/mL (STAT) and ~10^5 CFU/mL (LTS) and held for 5, 10, 15, and 20 minutes before removing samples for dilution in 0.1% (w/v) peptone and subsequent plating on agar media. The same procedure was repeated for the apple juice with added 0.05% cinnamaldehyde.

Measurement of pH and degrees BRIX

Measurements of pH of non-inoculated saline or apple juice with or without added cinnamaldehyde were performed using an Orion Model 525 pH meter (Orion Research, Inc., Boston, MA) fitted with a glass electrode. Prior to performing the pH measurements, all samples of saline and apple juice were tempered to 22 ± 1 ^{o}C. Measurements of degrees BRIX of apple juice were performed using a digital Pocket Refractometer PAL (ATAGO, USA, Inc., Bellevue, WA).

Microbiological analysis

At the set time intervals (5, 10, 15 and 20 min), 1-mL aliquots from the inoculated saline or apple juice were added to tubes containing 9 ml of 0.1% (w/v) peptone and 10-fold serial dilutions were performed. Aliquots (0.1-mL) of appropriate dilutions were surface-plated in duplicate on XLT4 and TSAYE for *S.* Typhimurium and on MOX and TSAYE plates for *L. monocytogenes* followed by incubation at 35^{0}C before counting bacterial colonies after 48h.

Calculation of D-values

The colony counts on TSAYE for STAT and LTS cells of both *S.* Typhimurium and *L. monocytogenes* and in saline and apple juice enumerated were used for preparing survivor curves to determine the decimal reduction times (D-values). Survivor curves were prepared by plotting

the number of culturable survivors (log_{10} CFU/mL) versus time using Microsoft Excel 2016 Software (Microsoft, Inc., Redmond, WA). That software program was used to generate the line of the best fit, perform linear regression analysis and determine the slopes of the survivor curves. The D-value is the negative reciprocal of the slope of the survivor curve and is therefore the time required for a specific concentration of cinnamaldehyde to kill 90% of the initial population of the target organism under the conditions described in the present study.

Evaluation of sub-lethal injury

Sub-lethal injury was evaluated using the mathematical model proposed by Wuytack et al. (2003). Using the raw data (CFU/ml) from colony counts on TSAYE and XLT-4 (for *S.* Typhimurium) and on TSAYE and MOX (for *L. monocytogenes*), the reduction factor (RF) and subsequently the log RF values for surviving populations on selective (XLT-4 and MOX) and non-selective media (TSAYE) were obtained at each sampling time via use of the following formula:

$$Log\ RF = Log\left(\frac{CFU\ before\ treatment(control)}{CFU\ after\ treatment}\right)$$

The log RF values for selective media were plotted on the y-axis and those from non-selective media on the x-axis in Microsoft Excel to form scatter plots from which slopes and intercepts were determined after linear regression analysis. The slopes from all the treatments and time intervals were compared to determine if each value was significantly different ($P<0.05$) from 1.0 and intercepts from 0 to identify treatments that resulted into significant levels of sub-lethal injury (Wuytack et al., 2003).

Statistical analysis

Each experiment was replicated three times and values representing reductions in viable counts (Log_{10} CFU/mL) of the STAT and LTS cell of the pathogens are reported as averages of the replicate trials. Significant differences in Log reductions and sub-lethal injury between LTS and STAT phase cells were determined using the Students t-test at a 5% significance level. Statistical tests were performed using JMP software (JMP version 14.2.0; SAS Institute, Cary, NC).

Results

Minimum inhibitory concentration and minimum bactericidal concentrations

The MICs of cinnamaldehyde for STAT cells of *S.* Typhimurium and *L. monocytogenes* in BHI broth were 0.22 and 0.1µL/mL, respectively. In saline, the MBCs of cinnamaldehyde for the LTS phase cells of both pathogens were higher than those for the STAT cells. The MBCs of cinnamaldehyde for STAT and LTS cells of *S.* Typhimurium were 0.45 and 0.90µL/mL respectively, whereas, the MBCs for STAT and LTS cells of *L. monocytogenes* were 0.30 and 0.60 µL/mL, respectively.

The pH and degrees BRIX

The average pH of the saline before and after addition of cinnamaldehyde was 5.70 and 5.64, respectively. The pH apple juice was 3.64 and 3.62 before and after addition of cinnamaldehyde (0.05%). The average Brix value for the apple juice was 11.7, which increased to 12.5 after addition of cinnamaldehyde.

Cell culturability in apple juice and saline

Figures 1 and 2 show the log reductions in initial populations of *S.* Typhimurium and *L. monocytogenes* following exposure to 0.1% cinnamaldehyde in saline (0.85% NaCl; 22 ± 1 °C) for up to 20 min and based on colony counts on non-selective agar (TSAYE). The Log reductions in

viability of STAT cells were significantly higher than those of LTS cells ($P< 0.05$). Increased exposure time to cinnamaldehyde resulted in increases in Log reduction for both pathogens with the greatest reduction in culturability occurring after 20 minutes. Exposure (20-min) of the pathogens to cinnamaldehyde in saline reduced initial populations of STAT *Salmonella* and *L. monocytogenes* by 6.0 and 6.29 Log, respectively. In contrast, LTS cells of those pathogens were reduced by 3.70 (*Salmonella*) and 2.73 (*L. monocytogenes*) Log. A similar trend was observed in apple juice whereby longer exposures to cinnamaldehyde resulted in greater Log reductions for both pathogens with the highest reduction in viability occurring after 20 minutes (Figures 3 and 4). For both pathogens the STAT cells were less tolerant to cinnamaldehyde compared to LTS cells irrespective of exposure time in the apple juice. After 20 min in juice with added cinnamaldehyde (0.05%) the initial populations of STAT *Salmonella* and *L. monocytogenes* were reduced by 4.76 and 5.25 Log, respectively whereas, reductions in viable counts of LTS cells were 3.33 (*Salmonella*) and 3.73 (*L. monocytogenes*) Log.

Resistance to cinnamaldehyde in saline and apple juice

Table 1 shows the D-values for STAT and LTS cells of *S.* Typhimurium and *L. monocytogenes* as influenced by cinnamaldehyde in 0.85% (w/v) saline and apple juice. The LTS cells of both pathogens exhibited significantly higher D-values compared to STAT cells in both saline and apple juice ($P<0.05$).

Sub-lethal injury in LTS and stationary phase cells

Table 2 shows slopes generated from linear regression plots derived from reduction factors of surviving populations of STAT and LTS cells of *S.* Typhimurium and *L. monocytogenes* in saline and apple juice with 0.1% and 0.05% added cinnamaldehyde, respectively. In saline, exposure of the pathogens to cinnamaldehyde resulted in significant sub-lethal injury in the

surviving populations of STAT cells ($P<0.05$). However, the extent of sub-lethal injury in populations LTS survivors of both pathogens in saline was not significant ($P>0.05$). In apple juice, significant sub-lethal injury was observed in STAT phase survivors of *L. monocytogenes* but not *S.* Typhimurium. Also, LTS survivors of both pathogens exhibited no significant sub-lethal injury in apple juice ($P>0.05$).

Discussion

Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

The minimum inhibitory concentration (MIC) is the lowest concentration of cinnamaldehyde at which no visible growth (turbidity) was observed in BHI (35 ^{o}C) after 24 h. In the present study, the higher MIC observed for STAT cells of *S.* Typhimurium compared to *L. monocytogenes* indicated a greater sensitivity of *L. monocytogenes* to cinnamaldehyde. In vitro studies on the antibacterial activity of several essential oils and essential oil components revealed that Gram-positive bacteria were more sensitive than Gram-negative bacteria (Burt, 2004). For instance, Chorianopoulos et al. (2004) reported that *Escherichia coli* and *Salmonella* Enteritidis were less sensitive to EOs compared to *Staphylococcus aureus*, *L. monocytogenes* and *Bacillus cereus*. Those findings as well as the MIC results of the present study agree with results reported by Sokovic et al. (2010) and Markelova & Semenova (2017). Those same authors demonstrated that Gram-negative bacteria survived better upon exposure to essential oils and their components. A plausible explanation for this observation is that the outer lipopolysaccharide membrane of Gram-negative bacteria that is absent in Gram-positive bacteria, restrict the entry of essential oils or essential oil components into the cell (Kim et al. 1995; Huisman *et al.,* 1996; Semeniuc et al., 2017; Thomas-Popo et al., 2019). While the sensitivity of STAT phase cells of the pathogens was evaluated via MIC tests, such evaluation was not feasible using LTS phase cells because growth of the target organism is crucial for determining the MIC of an antimicrobial. Considering that the

LTS phase cells are dormant (Wen et al., 2009; Wang et al., 2018) their growth in broth would indicate that they have "broken" dormancy and exited from the LTS phase of the bacterial life cycle. Consequently, the MIC results will be meaningless with respect to LTS cells.

The MBC is the lowest concentration of an antimicrobial that results in ≥99.9% (3 log) kill of the initial population of the target organism is the MBC (CLSI, 1999; García-García et al., 2011; Calo et al., 2015). In the present study, we determined the MBC of cinnamaldehyde for the target pathogens (in STAT and LTS phases) after 24h in saline (35^0 C). The LTS phase cells for both pathogens were more tolerant to cinnamaldehyde compared to the STAT cells, with *S.* Typhimurium exhibiting even more tolerance to compared to *L. monocytogenes*. Our observations that the MBC values for the LTS phase cells for *S.* Typhimurium and *L. monocytogenes* were higher than those for STAT phase cells indicate the stronger resistance of LTS cells to cinnamaldehyde.

Two different concentrations of cinnamaldehyde (0.1% in saline and 0.05% in apple juice) were used to determine the extent to which STAT and LTS cells of both pathogens showed resistance to that EO component at bactericidal concentrations. Preliminary testing of 0.1% cinnamaldehyde resulted in drastic reductions of initial populations of STAT cells in apple juice (pH 3.62); no survivors of STAT *L. monocytogenes* and *S.* Typhimurium could be detected by plating juice samples on TSAYE after 15 and 20 minutes, respectively. This led to the decision to use a lower concentration of cinnamaldehyde (0.05%) for subsequent experiments with apple juice. It is well known that essential oils (EO) and EO components such as cinnamaldehyde, eugenol, thymol, and carvacrol display increased antimicrobial potency under low pH or acidic conditions. Lower pH increases the hydrophobicity of the EO components, resulting in greater antimicrobial efficacy due to greater interactions with cell membrane lipids to ultimately cause

severe injury or kill the bacterial cell (Negi, 2012; Mendonca *et al*., 2018; Thomas-Popo *et al.*, 2019).

Cell culturability in apple juice and saline

Several published studies have reported on the inactivation of enteric pathogens by cinnamaldehyde in apple juice or apple cider (Baskaran et al., 2010; Friedman et al., 2004) and other fruit juices such as watermelon (Siddiqua et al., 2015), carrot broth (Hernandez-Herrero et al., 2008), carrot juice and mixed berry juice (Manu et al., 2017) and tomato juice (Friedman et al., 2017). In all instances those authors used stationary phase (STAT) cells for evaluating the antimicrobial efficacy of cinnamaldehyde. The STAT cells have a very long history of use in microbiological experiments involving testing of antimicrobial agents. Also, STAT cells are far more resistant to antimicrobial agents compared to exponential phase cells (Kolter et al., 1993; Pletnev et al., 2015; Rees et al., 1995). Based on those facts, the belief that STAT cells are ideal use in process validation studies to evaluate effectiveness of antimicrobial agents is almost scientific dogma in food microbiology.

In the present study, we compared STAT and LTS cells of *S.* Typhimurium and *L. monocytogenes* for their tolerance to cinnamaldehyde in saline and apple juice. In this regard, the LTS cells consistently exhibited significantly higher tolerance to cinnamaldehyde ($P<0.05$) in both media (Figures 1 to 4). Other researchers have reported significantly higher resistance of LTS cells of pathogens to physical antimicrobial processes. For example, Wang et al. (2018) demonstrated that LTS cells of *S.* Typhimurium ATCC 14028 were more resistant than exponential- and STAT phase cells to ultraviolet light in 0.85% saline and apple juice ($P<0.05$). The LTS cells of *L. monocytogenes* ATCC 19115 exhibited significantly ($P<0.001$) greater tolerance to high pressure and heat than exponential or STAT cells in UHT whole milk (Wen et

al., 2009). Those results indicate the high potential of LTS cells of pathogenic bacteria to survive antimicrobial interventions that would otherwise eliminate cells in the stationary phase of the life cycle. Therefore, conservative approaches to developing ways to inactivate foodborne pathogens should involve the use of target organisms in the LTS phase.

While the four phases of the bacterial life cycle namely, lag, exponential, stationary and death phase, are relatively well known in microbiology, the fifth phase (LTS) has often been overlooked. The LTS phase occurs after the death phase in which about 94% of the cell population die (Wen et al., 2009). This often-neglected phase has been reported in *S.* Typhimurium (Wang et al., 2018; Djebbi-Simmons et al., 2019), *L. monocytogenes* (Wen et al., 2009) and other bacteria (Finkel, 2006; Lappin-Scott and Costerton, 1990) where the cells transform from rods to cocci and can persist for months or years in a seemingly dormant state. During their transition to the LTS phase cells seem to develop resilient characteristics that help them to withstand various physical or chemical stresses, including cinnamaldehyde as observed in the present study. While the precise mechanism of antibacterial action of cinnamaldehyde is unclear, it is believed that this EO component causes inactivation of bacterial cells by inhibiting ATPase and thus energy production at sub-lethal concentrations and disrupting the cytoplasmic membrane at lethal concentrations (Hyldgaard et al., 2012). Gill and Holley (2006) demonstrated that when *Escherichia coli, L. monocytogenes* and *Lactobacillus sake* were exposed to bactericidal concentrations of cinnamaldehyde, the primary cellular target for their inactivation was the cytoplasmic membrane. In this regard, the highly significant upregulation of genes for cell envelope structure and energy metabolism observed in LTS cells (Wen et al., 2011) might be responsible for greater tolerance of LTS cells of both *S.* Typhimurium and *L. monocytogenes* to cinnamaldehyde at concentrations used in the present study.

Resistance of LTS and STAT cells in cinnamaldehyde

The D-value is defined as the time required for the selected concentration of cinnamaldehyde to achieve a 1.0 log reduction of either pathogen in its respective phase of the life cycle under the test conditions described in the present study. Therefore, the higher the D-value, the greater is the resistance of the target organism. The higher D-values observed in LTS cells of both pathogens imply that LTS cells were more tolerant to cinnamaldehyde compared to STAT cells irrespective of whether they were in saline or apple juice. These findings provide further evidence in support of the greater resistance of LTS cells to cinnamaldehyde, an EO component with demonstrated activity against both Gram-negative and Gram-positive bacteria (Gill and Holley, 2004). Considering that EO components such as cinnamaldehyde are of interest for the development as antimicrobial food additives, it is crucial that evaluation of antibacterial activity include the use of LTS cells as target organisms to ensure food safety.

Sub-lethal injury in LTS and stationary phase cells

Determination of sub-lethal injury in foodborne pathogens following their exposure to physical or chemical antimicrobial agents is important for two main reasons. Firstly, the presence of sub-lethally injured pathogens in foods can falsely overestimate the antimicrobial effectiveness of the antimicrobial treatment. This is especially true if selective culture media are used to determine numbers of pathogen survivors. Secondly, antimicrobial agents that inflict sub-lethal injury in pathogens allow for their combined use with other antimicrobial interventions to prevent cellular repair and subsequent proliferation of sub-lethally injured pathogens. This can result in death of those pathogens to substantially improve the microbial safety of food products.

We evaluated sub-lethal injury in STAT and LTS cells of *S.* Typhimurium and *L. monocytogenes* by plotting the logarithm of the calculated viability reductions for the selective agar (XLT-4 or MOX) on the y axis versus the logarithm of viability reductions for TSAYE on

the x axis and fitting linear regression lines through the data points. The extent sub-lethal injury caused by cinnamaldehyde was evaluated from the slopes and y intercepts of the regression lines (Wuytack et al., 2003). There is no sub-lethal injury when a slope of 1.0 and intercept of 0 are observed because the same viability reduction occurs on both the selective medium (XLT-4 or MOX) and on the non-selective medium (TSAYE). When a slope is significantly greater than 1.0 or an intercept is significantly greater than 0, there is sub-lethal injury due to the higher viability reduction noted on selective medium than on the non-selective medium. Additionally, a greater deviation of the slope from 1.0 or the intercept from 0 is indicative of a larger extent of sub-lethal injury. In the present study, the slopes of the regression lines for viability reduction of STAT cells of the pathogens in saline containing 0.1% cinnamaldehyde were significantly greater than 1.0 indicating sub-lethal injury in the surviving population ($P<0.05$). These results are in contrast to those for viability reduction in LTS cells which exhibited no significant sub-lethal injury ($P>0.05$). Generally, that same trend was observed in apple juice containing 0.05% cinnamaldehyde where the extent of sub-lethal injury in LTS cells of the pathogens was not significant ($P>0.05$). Our observation that no significant sub-lethal injury occurred in STAT survivors of *S.* Typhimurium in apple juice warrants further research. Overall, these findings indicate that, compared to LTS cells, a significantly greater extent of sub-lethal injury occurred among STAT survivors following exposure of the pathogens to cinnamaldehyde.

Conclusions

The LTS cells of both *S.* Typhimurium and *L. monocytogenes* are significantly more tolerant than STAT cells to cinnamaldehyde in saline and apple juice. The significantly higher decimal reduction times (D-values) for LTS cells and low level of sub-lethal injury in LTS survivors attest to the substantially greater resistance of LTS pathogens to inactivation by

cinnamaldehyde. To further ensure microbial food safety, LTS cells of foodborne enteric pathogens should be employed as the target organisms in the development of antimicrobial processes involving addition of cinnamaldehyde in food products.

Disclosure statement

There were no competing financial interests in this study.

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Figures and Tables

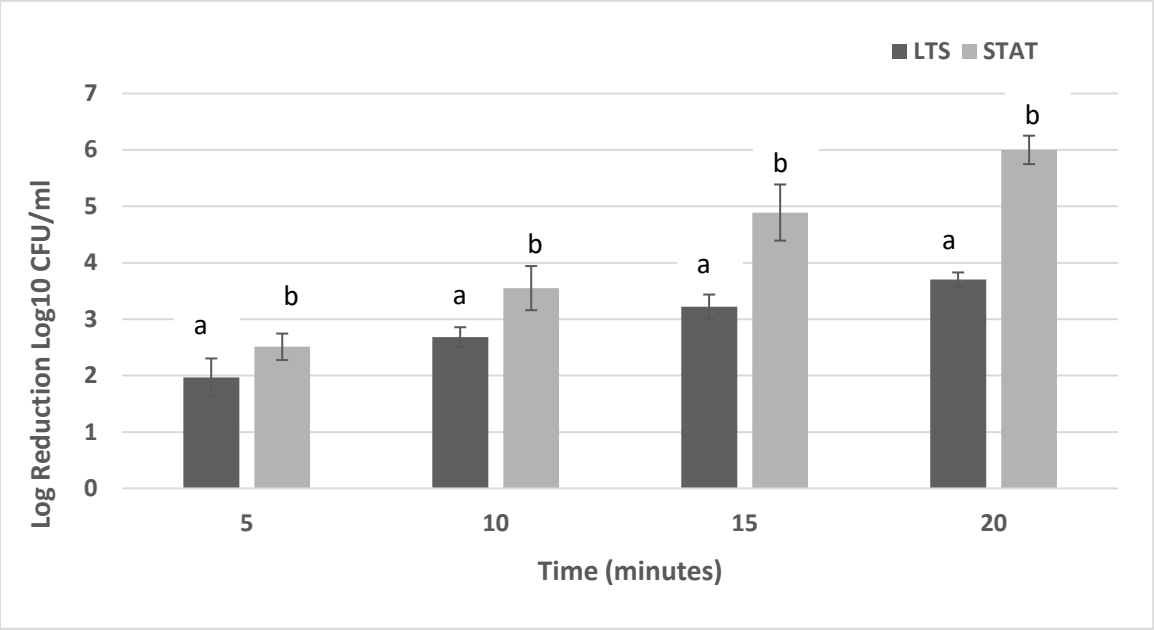

Figure 1. Log reductions (CFU/mL) of initial populations LTS and STAT cells of *Salmonella* Typhimurium ATCC 14028 in saline with 0.1% cinnamaldehyde. For each time point, bars with different letters are significantly different (P< 0.05).

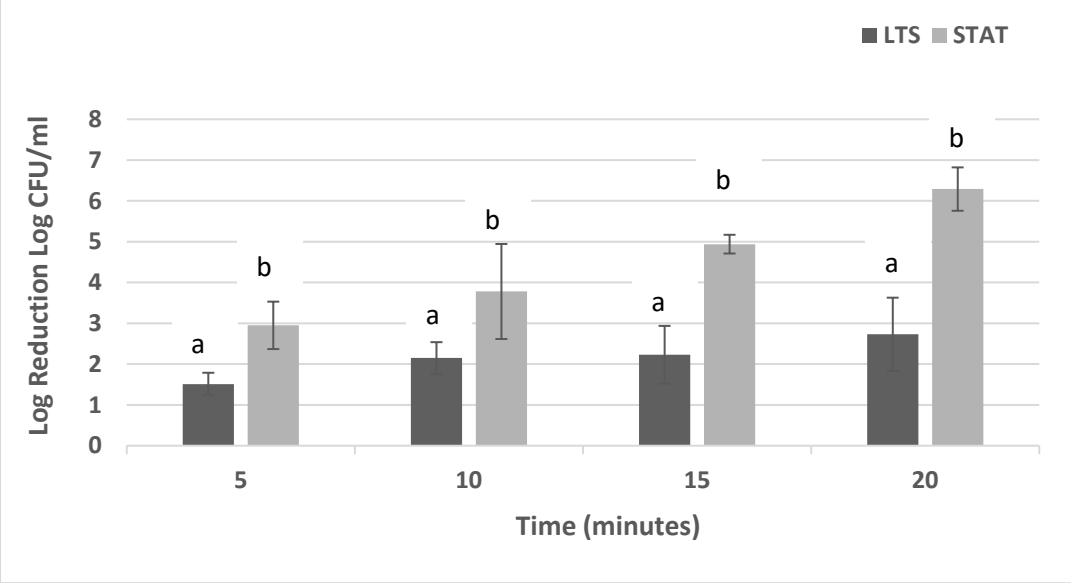

Figure 2. Log reductions (CFU/mL) of initial populations LTS and STAT cells of *Listeria monocytogenes* in saline with 0.1% cinnamaldehyde. For each time point, bars with different letters are significantly different (P< 0.05).

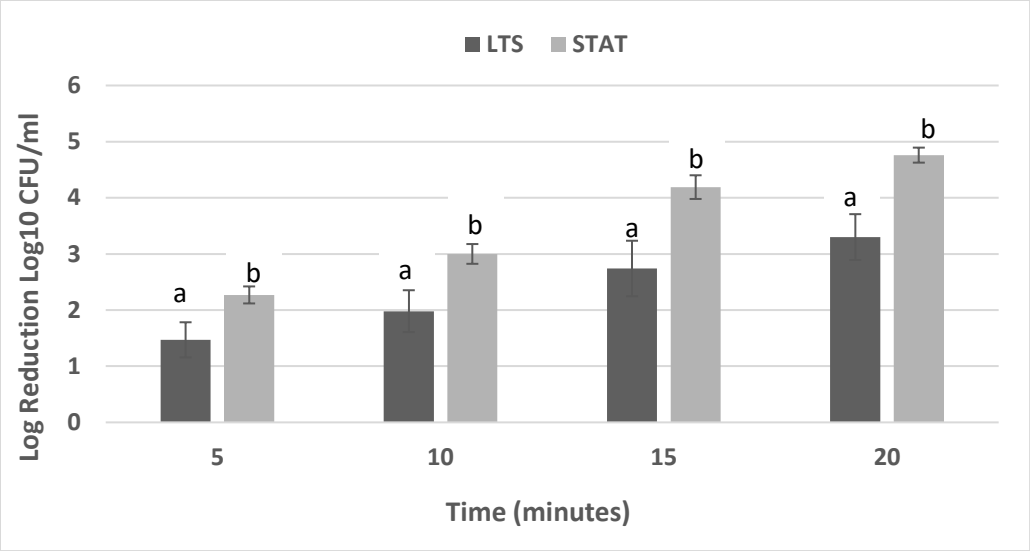

Figure 3. Log reductions (CFU/mL) of initial populations LTS and STAT cells of *Salmonella* Typhimurium in apple juice with 0.05% cinnamaldehyde. For each time point, bars with different letters are significantly different (P< 0.05).

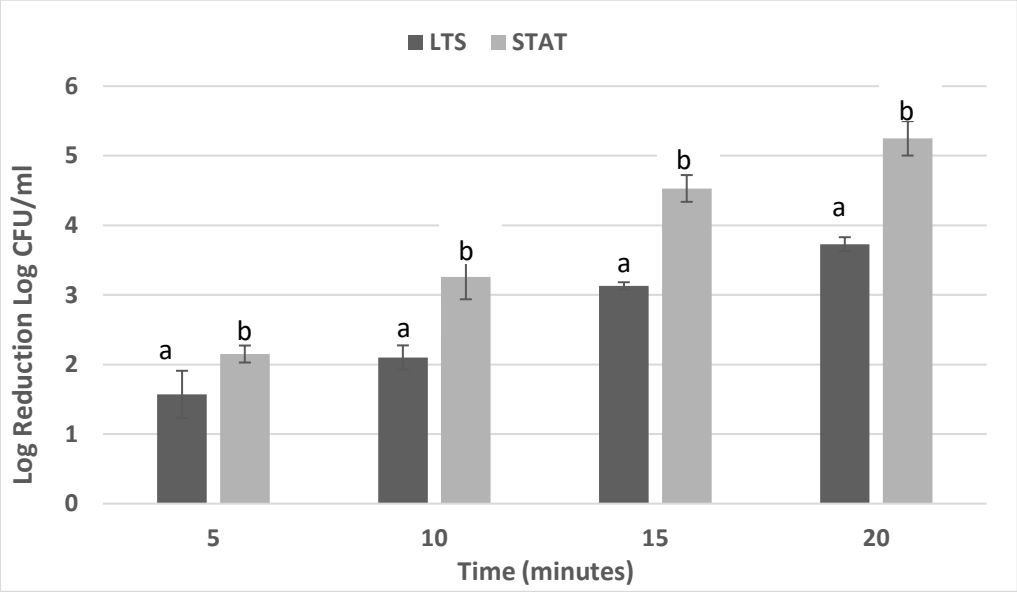

Figure 4. Log reductions (CFU/mL) of initial populations LTS and STAT cells of *Listeria monocytogenes* in apple juice with 0.05% cinnamaldehyde. For each time point, bars with different letters are significantly different (P< 0.05).

Table 1. D-values (minutes) for Stationary and Long-Term Survival phase cells of *Salmonella* Typhimurium and *Listeria monocytogenes* in saline and apple juice with 0.1% and 0.05% added cinnamaldehyde, respectively

Pathogen	Life cycle phase	0.85% saline + 0.1% CIN	Apple juice + 0.05% CIN
S. Typhimurium	STAT	3.49± 0.19 A	4.47± 0.72 A
	LTS	5.81± 0.42 B	6.47± 0.86 B
L. monocytogenes	STAT	3.45± 0.27 A	3.89± 0.18 A
	LTS	6.38± 0.15 B	5.48± 0.29 B

The data used to calculate the D-values were derived from bacterial counts of survivors on non-selective media agar (tryptic soy agar supplemented with 0.6% yeast extract). Values are averages ± standard deviation from three replicate experiments. For each pathogen, averages with different letters within each column are significantly different ($P<0.05$). STAT, stationary. LTS, long-term survival. CIN, cinnamaldehyde.

Table 2. Slopes and intercepts from plots showing reduction in viability for *Salmonella* Typhimurium and *Listeria monocytogenes* in saline and apple juice with added cinnamaldehyde at 0.1% (A) and 0.05% (B), respectively.

(A) **Pathogen**	**Life cycle phase**	**Slope**	**Intercept**	**R-square**
S. Typhimurium	STAT	1.48±0.08[a]	-0.67±0.04	0.9239
	LTS	0.97±0.02	-0.33±0.09	0.8842
L. monocytogenes	STAT	1.63±0.07[a]	-0.73±0.35	0.8945
	LTS	1.00±0.05	-0.79±0.16	0.9423
(B) **Pathogen**	**Life cycle phase**	**Slope**	**Intercept**	**R-square**
S. Typhimurium	STAT	1.17± 0.02	-0.825±0.06	0.9695
	LTS	0.98± 0.04	-0.79±0.16	0.9756
L. monocytogenes	STAT	1.36 ± 0.04[a]	-0.81±0.27	0.9539
	LTS	1.16 ± 0.18	-1.02±0.10	0.9932

[a] Slope is significantly different from 1.0 ($P<0.05$)
[b] Intercept is significantly different from 0 ($P<0.05$)

CHAPTER 5. GENERAL CONCLUSIONS

The following conclusions are made based on the research reported in the present thesis:

Salmonella enterica can survive for eight hours (and possibly more) on chicken breast meat marinated in lemon juice; therefore, the use of only lemon juice as marinade solution might not completely eliminate this pathogen from raw chicken meat.

Over time, the pH of lemon juice marinade containing raw chicken meat increases and this change can potentially allow increased survival of *Salmonella enterica* in the marinade solution.

Salmonella enterica cells inoculated onto chicken breast and allowed to attach to the meat surface are less sensitive to the killing effect of lemon juice compared to cells inoculated directly into the lemon juice.

Yucca extract can serve as an effective natural emulsifier to aid the dispersion of thyme oil in lemon juice marinades.

Thyme oil has good potential to enhance the antimicrobial efficacy of lemon juice against *Salmonella enterica* on raw chicken meat during marination.

In apple juice, the long-term survival cells of both *Salmonella* Typhimurium and *Listeria monocytogenes* are more resistant to cinnamaldehyde than stationary phase cells of those same pathogens.

The greater resistance of long-term survival phase cells of foodborne enteric pathogens to cinnamaldehyde position them as suitable the target organisms for use in validating antimicrobial processes involving addition of cinnamaldehyde in juices to enhance food safety.