EVALUATION OF THE EFFICACY OF ANTIMICROBIAL AGENTS TO PREVENT THE TRANSFER OF *LISTERIA MONOCYTOGENES* FROM BIOFILMS TO PRODUCE OR PROCESSING SURFACES

by

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This thesis is dedicated to my children for their unconditional love.

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ABSTRACT

Bacteria that are part of a biofilm community are considerably less susceptible to antimicrobial agents than their planktonic counterparts. It is therefore very difficult to eradicate biofilm-associated bacteria from surfaces of fruits and vegetables or harvesting and processing surfaces. Biofilm-associated bacteria surviving treatments with antimicrobials can be released from the biofilm and colonize other surfaces.

This research focused on biofilms consisting of either a mixture of six *Listeria monocytogenes* (*Lm*) isolates or a mixture of the *Lm* isolates and four stains isolated from surfaces at a produce processing plant (PPPI). The isolates were identified by 16S rRNA sequencing as belonging to the genera *Acinetobacter, Pedobacter, Chryseobacterium* and *Pantoea*. The efficacy of sodium hypochlorite (NaOCl) and peroxyacetic acid (PAA) in preventing *Lm* released from biofilms to take refuge on fresh produce (spinach) or processing surfaces was studied. Pure *Lm* and multi-species biofilms were grown on flat bottom 96-well microtiter plates or stainless coupon surfaces and the effectiveness of the antimicrobials dissolved in tap water in preventing the transfer of *Lm* to polystyrene pegs (sterile and non-sterile surfaces) or fresh produce (spinach) was determined.

Complete inactivation of *Lm* in a mono-species biofilm on Calgary Biofilm Device (CBD) pegs required a 30-min exposure to 50 ppm or a 7.5-min exposure to 100 ppm NaOCl in tap water. *Lm* in mixed species biofilms were inactivated after a 30 min exposure to 25

ppm or 7.5 min exposure to 250 ppm of NaOCl in tap water. PAA achieved complete inactivation of pure *Lm* species biofilm at a concentration of 120 ppm and 15 ppm for 7.5 and 30 min, respectively. When present in multispecies biofilms, *Lm* was inactivated when exposed to 240 ppm for 7.5 mins or 120 ppm for 30 mins. *Lm* in mixed-species biofilms enjoyed a higher level of protection from antimicrobials than *Lm* in pure biofilms. Also, side-by-side comparison of the effects of different concentrations of PAA or NaOCl and exposure times indicated that *Lm* added to existing biofilms was about as sensitive to the anti-microbial as *Lm* that was part of the biofilm from the start.

Transfer of *Lm* from existing biofilms was prevented by NaOCl at concentrations between 25 and 50 ppm and 50 and 100 ppm for sterile and non-sterile surfaces, respectively. The concentration of PAA that achieved the same effect was between 7.5 and 120 ppm and 60 and 240 ppm for sterile surfaces and non-sterile surfaces, respectively. Prevention of transfer of *Lm* from a biofilm to spinach was achieved at concentrations of NaOCl lower than 20 ppm; however, even the highest concentration of NaOCl (250 ppm) applied was not sufficient to completely inactivate the residual *Lm* on the stainless coupons after a 20-min exposure time. The information gathered from this study highlights the need of produce processors to make every effort to prevent the development of *Lm*-containing biofilms and to carefully monitor the concentration of the antimicrobials used throughout the wash phase.

Chapter 1

INTRODUCTION

1.1 Biofilms

Biofilms are microbial communities that grow attached to surfaces, are usually embedded in self-produced organic polymeric matrices commonly known as extracellular polymeric substances (EPSs), and present complex three-dimensional architectures (Puga, SanJose, & Orgaz, 2016; Mosquera-Fernandez et al., 2016). EPSs provide structural integrity to biofilms and some level of protection to the microorganism against environmental influences such as chemical disinfecting agents, UV radiation and antibiotics (Purkrtová et al., 2010; Stiefel et al., 2016). The significance of biofilms and their ubiquity have gradually emerged since their first description (Costerton et al., 1995). In the environment, biofilms are frequently formed by different species that inhabit the same niche (Costerton et al., 1995).

1.2 Listeria monocytogenes

Listeria monocytogenes (*Lm*) is a gram-positive pathogenic bacterium which is ubiquitous in the environment. It can persist in food processing facilities for extended periods of time due to its ability to form biofilms, likely with other bacteria found within the processing area, and can eventually be transferred from food contact surfaces to food (Rodrigues et al., 2011). Consumption of fresh produce contaminated with *Lm* can cause listeriosis in susceptible individuals. This disease mostly affects immuno-compromised persons, neonates, pregnant women, elderly persons and AIDS patients (Todd & Notermans, 2011). It is characterized by various clinical conditions such as spontaneous abortions, meningoencephalitis, septicemia, gastroenteritis and serious infections to the newborns. Every year 1600 people are infected in the US and about 260 succumb to the disease (Hernandez-Milian & Payeras-Cifre, 2014). Although its incidence is relatively low (EU notification rate was 0.46 cases per 100,000 population for 2015), its case fatality rate has been the highest one for illnesses caused by food borne pathogens, at 17.7% (Prevention, 2016).

1.3 *Listeria monocytogenes* in the produce industry

Minimally processed ready-to-eat fruits and vegetables may contain *Lm* among their microbiota owing to contamination at some point in the process from

cultivation to consumption (Nyarko et al., 2016). Treatment of fresh produce and of contact surfaces with antimicrobial agents is therefore done by many produce processors as ready to eat fresh produce does not undergo heat treatment (pasteurization) that would inactivate most bacteria including *Lm* before consumption (Doijad et al., 2015; Montgomery & Banerjee, 2015). Nevertheless, produce-related listeriosis outbreaks in the US and around the world have occurred on multiple occasions (*Table 1).* In addition to the public health concern, listeriosis outbreaks also lead to huge economical losses as the company concerned is obligated to recall the product. Various reports have shown that produce-related outbreaks in the US have been declining since the most severe outbreak in 2011 (*Figure 1).* The explanation for this decline is attributed to companies adopting better sanitation practices and making improvements to existing ones.

The major reason for the contamination of industrially processed produce appears to be due to the persistence of *Lm* in the produce processing environment (Colagiorgi et al., 2017; Kornacki, 2004). *Lm* entry points may be several but one major route could be through soil attached to produce entering the processing plant since this pathogen is present in soils (McCollum et al., 2013b). It then finds harborage sites which include, floor drains, crevices, walls especially cracks that retain moisture, trolleys, forklifts, maintenance tools, equipment framework ranging from rotating blades to belts and conveyors (Suslow & Harris, 2000). *Lm* persistence in food processing facilities, including those handling produce is

facilitated by its ability to grow at a wide pH (2.5-10) and temperature range (0-45^{0}C) (Campdepadrós et al., 2012), and at relatively high salt concentrations (10-20% sodium chloride). *Lm* can also withstand a certain level of stress caused by acidic conditions, chemical disinfectants, cold and heat shocks (Doijad et al., 2015; Swaminathan & Gerner-Smidt, 2007; Xia et al., 2016). Whether or not *Lm* by itself can form true biofilms is a matter of debate since, aside from sialic acid which is always present on the cell surface of *Lm*, no EPSs have been found so far (Colagiorgi et al., 2016; Doijad et al., 2015). Nevertheless, *Lm* is capable of readily attaching to and multiplying on various surfaces (Alonso et al., 2014; Midelet & Carpentier, 2002).

Interventions leading to the inactivation of *Lm* in these biofilms would be highly desirable, but numerous studies have shown that cells in such biofilms are very resistant to antimicrobial agents and that concentrations of antimicrobials able to kill planktonic cells are insufficient to inactivate biofilm cells. For instance, in a study comparing the efficacy of ozone, chlorine and hydrogen peroxide in eliminating *Lm* from biofilms, a concentration of these antimicrobials that was multiple times higher than that required for inactivation of planktonic cells was necessary (Purkrtová et al., 2010; Robbins et al., 2005 ; Rodrigues et al., 2011). In another study, the activity of sodium hypochlorite, iodine, biguanide, quaternary ammonium compounds and peracetic acid against *Lm* cells in suspension and adhered to stainless steel surfaces was investigated. Scanning electron microscopy

observations revealed that *Lm* cells adhered to surfaces after treatment with the disinfectants. It was found that the number of viable cells of *Lm* biofilm compared to the positive control (~6.2 Log CFU/ cm^2) were lower after treatment for 10 minutes with sodium hypochlorite (~1.0 Log CFU/cm^2) and peracetic acid (~1.1 Log CFU/cm^2) than with biguanide (~3.0 Log CFU/$cm^{2)}$. However, no viable planktonic cells were observed following the antimicrobial treatments (Cabeça et al., 2012).

Yet, in another study investigating the resistance of *Lm* biofilms to sanitizing agents in a simulated food processing environment, it was observed that *Lm* cells were reduced in number following antimicrobial treatments to biofilms grown on stainless steel coupons. However, repeated exposure to peroxide as well as other sanitizers (QACs and chlorine) led to development of resistance (Pan et al., 2006).

Based on these examples, it was concluded that *Lm* biofilm resistance to antimicrobial agents is a multifactorial process resulting from different mechanisms and causing the inefficiency of disinfectants even at the usable concentrations of commercial solutions (Bridier et al., 2011). Therefore, control strategies are needed to overcome these limitations. Prevention of transfer of *Lm* cells from existing biofilm to produce or other surfaces could be one intervention that might help improve the anti-biofilm strategies currently in use.

Table 1. Produce related outbreaks in the USA and around the world

Outbreak Location/ Year	Illness Cases	Deaths	Food Vehicle	Recall	Reference
Boston, USA, 1979	20	3	Raw vegetables	Unknown	(Ho, Shands, Friedland, Eckind, & Fraser, 1986)
Nova Scotia, Canada, 1981	41	17	Coleslaw	YES	(Zhu, Gooneratne, & Hussain, 2017)
Moncalieri and Giaveno, Italy, 1997	2930	0	Corn	Unknown	(Aureli- et al., 2007)
Virginia, USA, 2008	20	0	Sprouts	YES	(Zhu et al., 2017)
Texas, USA, 2010	10	5	Celery	YES	(Zhu et al., 2017)
Colorado, USA, 2011	147	33	Cantaloupes	YES	(McCollum et al., 2013a)
Illinois& Michigan, USA, 2014	5	2	Mung bean sprouts	YES	(Garner & Kathariou, 2016)
California, USA, 2014	35	7	Caramel Apples	YES	CDC
Ohio, USA, 2015	19	1	Lettuce	YES	(Jackson et al., 2016)
Washington, USA, 2016	9	3	Frozen Vegetables	YES	CDC

Food regulatory agencies such as the US FDA and the European Food Safety Authority (EFSA) consider *Lm* as a food adulterant and have enacted a 'zero tolerance' policy (FDA, 2016). Producers of RTE foods such as fruits and vegetables are required to recall any product found to be contaminated with the

pathogen or if it was previously isolated from the processing plant environment. In 2010, the Texas Department of State Health Services (DSHS) reported a listeriosis outbreak linked to chopped celery. Of the 10 people infected aged between 56 - 93 years, five died within three months (Zhu et al., 2017). During a 2011 outbreak, 147 people in 28 States became ill and 33 died (McCollum et al., 2013a). The source of *Lm* involved in this outbreak was identified as cantaloupes processed at a facility in Colorado since pulsed- field gel electrophoresis (PFGE) patterns from *Lm* isolated from those cantaloupes and from the patients' blood matched. More recently, a multistate outbreak of *Lm* linked to frozen vegetables originating from Washington State affected 9 people of which 3 died (CDC, 2017).

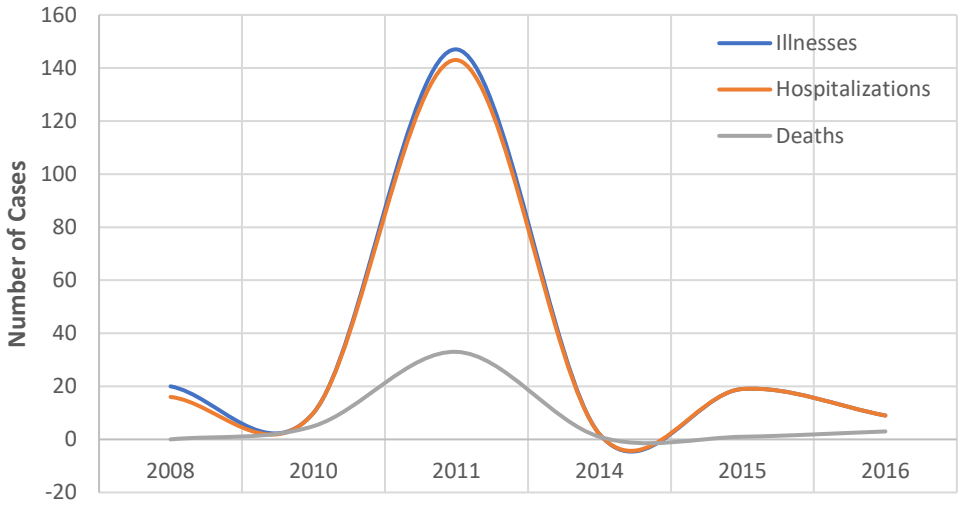


Figure 1. Illnesses, hospitalizations and fatalities due to produce-related listeriosis infections in the USA

1.4 Combating *Lm* with antimicrobials

1.4.1 Chemical antimicrobials

Studies with *Lm* in biofilms have routinely shown that it is difficult or impossible to achieve 5-log reductions of *Lm* in biofilms with various antimicrobials including sodium hypochlorite, chlorine dioxide, electrolyzed water, peroxyacetic acid, ozone, quaternary ammonium compounds, organic acids, pulsed ultraviolet light and trisodium phosphate (Choi et al., 2012; Kostaki et al., 2012; Montgomery & Banerjee, 2015; Ölmez & Temur, 2010; Hülya et al., 2009; Sagong et al., 2011; Hoelzer et al., 2012). While it is possible to utilize concentrations of some antimicrobials that will completely inactivate biofilm-bound *Lm* on food contact surfaces, the use of such high concentrations may be illegal or simply undesirable when in direct contact with food due to health concerns or damage to food quality or equipment. Thus, it is possible and perhaps likely, that *Lm* in biofilms on produce, surfaces in direct contact with produce or on soil particles cannot be inactivated by current and FDA approved antimicrobial rinses.

Approved chemical agents for use as disinfectants by the FDA include potassium, sodium or calcium hypochlorite whose direct application to food contact surfaces should not exceed 200 parts per million (ppm), peroxyacetic acid (PAA) (100-200 ppm), acetic acid (150-300 ppm), hydrogen peroxide (550-1100 ppm), chlorine dioxide (100-200 ppm), quaternary ammonium compounds (QACs) (150-400 ppm)

among others. The criteria for choosing which sanitizer to use by a given food processing company is based on nature of the company and the branding. For instance, pure organic produce processors will employ peroxyacetic acid and hydrogen peroxide whereas regular non-organic producers will employ the others such as sodium hypochlorite, QUATs, chlorine dioxide or a combination of the three in addition to others. The organoleptic quality of a product is another factor as some of the sanitizers may influence the final flavor, taste and color of the final produce. Other factors include, cost of sanitizer, ease of application and public safety (US Food and Drug Administration, 2017).

1.4.2 Alternatives to chemical treatments for *Lm* reduction

The inability of most chemical sanitizers to completely inactivate *Lm* cells in biofilms at currently recommended or commonly used concentrations has made *Lm* a growing concern in the food industry. This phenomenon as well as concerns about health or environmental effects of the chemicals has prompted research activities regarding the use of biological agents such as phages and enzymes as alternatives to chemical and physical methods of controlling *Lm* in biofilms. P100 was the first phage preparation for use against *Lm* that received GRAS status and approval in 2006 (FDA, 2006). As of now, one additional *Lm* phage preparations, ListShield^{TM}

has been approved by the Food and Drug Administration (FDA, 2014);however, no data on actual use of these products in the produce industry are available.

Furthermore, enzymes that act on the biofilm matrix are being explored as a mean to dislodge *Lm* from the biofilm matrix and from surfaces and expose it to a subsequent inactivation treatment. In a recent study, Rodríguez-López Jet al., 2017) assayed the effects of pronase, cellulase and DNase I alone or combined with benzalkonium chloride (BAC) against *Lm*-carrying biofilms. They observed that the best removal activity against *Lm*-*E. coli* dual species biofilms was obtained using DNase I followed by pronase and cellulase. Also, a higher number of released viable cells was observed after combined treatments compared to using a BAC only treatment. The results of this study open new perspectives for enzymes as an anti-biofilm strategy for *Lm* biofilm control; however, only a few enzymatic sanitizers are commercially available, possibly because the observations made with enzymes in laboratory settings often cannot be made in practice (Arya & Agarwal, 2005; Stiefel et al., 2016).

1.5 Research focus and objectives

Due to its low cost, sodium hypochlorite (bleach) is probably the most common antimicrobial added to fresh produce rinses. Along with peroxyacetic acid, it is also approved as a treatment agent for organic produce (US Food & Drug

Administration, 2017). This study therefore focused on these two antimicrobial agents with an aim of determining if they are able to inactivate *Lm* cells released form biofilms and prevent the transfer of *Lm* from the biofilms to other surfaces or fresh produce. The Calgary Biofilm Device (CBD) model system allows high throughput determination of Minimum Biofilm Eradication Concentrations (MBECs) for a wide range of antimicrobial agents. The study utilized *Lm*-only and multispecies biofilms grown on the surfaces of 96-well microtiter plates and subjected the biofilms to treatments with varying concentrations of sodium hypochlorite or peroxyacetic acid. The ability of these antimicrobial agents to prevent the transfer of *Lm* released from the biofilms in the presence or absence of organic matter (soil or sand) to new surfaces was also assayed by lowering sterile pegs of the CBD into the treatment solutions of sodium hypochlorite or peracetic acid. After completion of the treatments, the pegs were assayed for the presence of *Lm*.

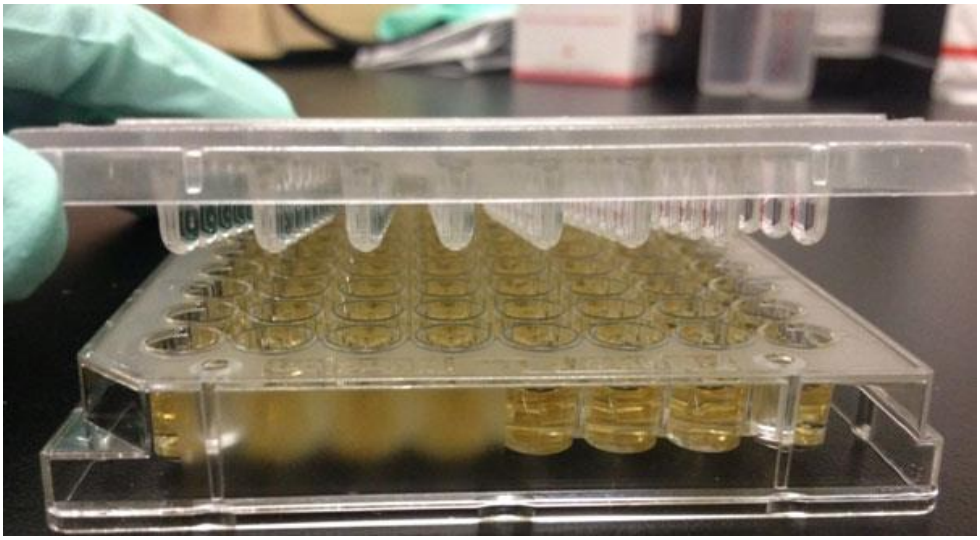


Fig. 2. Calgary Biofilm Device (CBD)

Specifically, the following objectives were addressed:

1. Determination of the biofilm-forming abilities of six *Lm* strains and four produce processing plant isolates (PPPIs).
2. Determination of the minimum biofilm eradication concentrations (MBECs) of sodium hypochlorite and peroxyacetic acid for *Lm.*
3. Determination of the efficacy of sodium hypochlorite or peroxyacetic acid to prevent transfer of *Lm* from existing biofilms to sterile surfaces (CBD pegs), surfaces colonized by bacteria other than *Lm* or fresh produce (spinach).

Chapter 2

BIOFILM FORMING ABILITIES OF SIX *LM* STRAINS AND FOUR PRODUCE PROCESSING PLANT ISOLATES (PPPIs)

2.1 Introduction

The major reason for the contamination of fresh produce and processing surfaces appears to be the persistence of *Lm* in the processing environment. The organism may enter the food processing environment through several routes and may establish a permanent presence at particular locations. Persistence of *Lm* is supported by factors such as growth at the wide pH range, salt tolerance, growth at low temperature, resistance to different stress conditions and the ability to attach to surfaces. . *Lm* has been shown to occur on surfaces that may directly or indirectly come in contact with food leading to contamination (Zhu et al., 2017).

Unlike strong biofilm formers such as many *Pseudomonas spp.*, *Lm* does not appear to produce biofilm-specific extra polymeric substances, although three-dimensional cell arrangements have been described (Doijad et al., 2015). Some scientists have questioned whether such surface-attached cell aggregates should be called “biofilms”, but since most researchers use this terminology, it will also be used in this thesis.

Several researchers have tried to relate *Lm* serotypes with their ability to adhere to surfaces, form biofilm, resist disinfectants and to tolerate stress; however, results remain contradictory or inconclusive (Borucki et al, 2003). In contrast studies have suggested that attachment to and growth on surfaces of *Lm* is strain-specific rather than serotype specific (Weiler et al., 2013). As a preliminary, this study aimed at assessing the biofilm forming abilities of the six *Lm* strains as well as the four PPPIs in microtiter plates under room temperature conditions.

2.2 Materials and Methods

2.2.1 *Listeria monocytogenes* strains

Lm Scott A is a widely used laboratory strain and is a serovar 4b. *Lm* 390-1 is one of the strains isolated during the investigations of the listeriosis outbreak associated with cantaloupes in 2011 (Goodridge, 2014). The other four strains (Table 2) were provided by Dr. Martin Wiedmann (Cornell University) and are part of a collection of strains suggested for use in food safety studies (https://foodsafety.foodscience.cornell.edu/research-and-publications/cps-strain-collection/). Stocks were maintained at −80 °C in tryptic soy broth (TSB) and 50% glycerol. To obtain working cultures, the strains were streaked onto Brilliance Listeria Agar (BLA) and a colony was inoculated into 2 ml of tryptic soy broth with 0.06% yeast extract (TSBYE) and incubated overnight at 37 °C with shaking.

Table 2. Lineage, serotype and isolation source of *Lm* strains from the strain collection maintained at Cornell University

ID	Lineage	Serotype	Source
FSL J1- 108	I	4b	Coleslaw, human, epidemic, Halifax 1981 (included to assure representation of a serotype 4b isolate)
FSL S10- 2161	II	Not available	Soil, spinach field
FSL J1- 107	I	4d	Human epidemic, coleslaw (includes to assure representation of a serotype 4d isolate)
FSL R9- 0506	II	1/2a	Cantaloupe 2011

2.2.2 Produce processing plant (PPPI) strains

2.2.2.1 Strain isolation and identification

Surfaces at a produce processing plant processing peaches and sweet corn were swabbed during operating hours and the swabs were streaked onto R2A medium. The plates were incubated at room temperature and one colony was picked from each plate for further purification by re-streaking. Purified colonies were grown in R2B medium at room temperature overnight and 250 µl of the culture was pelleted by centrifugation, resuspended in sterile deionized water and the suspension was

boiled for 5 min in a water bath. The samples were centrifuged at 10,000 g for 1 min and 2 µl of the supernatant was used for PCR to amplify 16s rRNA genes using primer pair 63f-1387r (Suzuki et al., 1996). The amplification products were precipitated with ethanol and subjected to cleavage with restriction enzyme *Hha*I. Four strains exhibited unique restriction enzyme patterns and their 16s rRNA gene amplification product were purified using the QIAquick® PCR purification kit (Qiagen)**.** Sequencing was done at the sequencing facility at the University of Delaware using primer 338F (5'ACTCCTACGGGAGGCAGC 3') (Takahashi et al., 1998). The sequences obtained were compared with sequences in GenBank using the Blast program (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The four strains from the produce processing plant were maintained at −80 °C in TSB and 50% glycerol. To obtain working cultures, the strains were streaked onto R2A and a colony was inoculated into 2 ml R2B medium and incubated at 22 °C with shaking overnight.

2.2.3 Biofilm development

The six *Lm* strains and the four-produce processing plant isolates (PPPI) were grown separately in R2B medium overnight at 30 °C. The optical density at 600 nm was determined for each culture by measurement of 100 µl of each culture in a 96-well microtiter plate in a microplate spectrophotometer (BIOTEK

Spectrophotometer). The cultures were diluted with R2B medium to achieve an optical density (OD) of 0.05 at 600 nm. Aliquots of 150 µl of each strain suspension were dispensed into microtiter plate wells in replicas of six for each strain. The microtiter plates were incubated at 22 ^{0}C to allow cell attachment and after 4 hours the suspension was removed and replaced with fresh R2B medium. Cells adhering to the microtiter surface were allowed to form biofilms over a period of 5 days with media replacement every 24 hours. The biofilm forming ability of the strains was assayed using the crystal violet test.

2.2.4 Crystal Violet Staining of biofilm

The staining procedure followed the protocol provided by (Stepanovic et al. (2004). The liquid was removed from the microtiter plate wells after 5 days of biofilm growth, the wells were rinsed three times with PBS to remove loosely attached cells and the wells were allowed to dry in an inverted position for 30 minutes. The biomass in the wells was stained by the addition of 150 µl of 0.1% CV crystal violet solution. The plates were sealed with parafilm and incubated for 45 minutes at room temperature. The wells were rinsed with PBS to remove unbound CV dye and the bound dye was solubilized by adding 200 µl of 95% ethanol. After incubation at 4 ˚C for 30 minutes, 100 µl of solubilized dye from each well was transferred to a fresh microtiter plate and the absorbance measured at 600 nm using 95% ethanol

as the blank. The OD of the ethanol blank (ODc) was subtracted from the sample blank to obtain the OD for classification of a strain with respect to its biofilm-forming ability. Based on the OD produced by bacterial films, strains were classified into the following categories; no biofilm formers, weak, moderate or strong biofilm producers as described by (Ranin et al., 2004). The cut-off O.D. (ODc) was defined as three standard deviations above the mean O.D. of the negative control. Strains were classified as follows: OD $\leq$ ODc = no biofilm producer, ODc < OD $\leq$ (2 × ODc) = weak biofilm producer, (2 × ODc) < OD $\leq$ (4 × ODc) = moderate biofilm producer and (4 × ODc) < OD = strong biofilm producer. All tests were carried out in triplicate and the results were averaged.

2.3 Results and Discussion

2.3.1 Identification of produce processing plant isolates (PPPI)

The 16S rRNA gene sequence entries in GenBank that most closely matched those of the four isolates from the produce processing plant are listed in Tables 3-6. The data indicated that isolates J1, I5, I3 and E3 belong to the genera *Acinetobacter, Pedobacter, Chryseobacterium* and *Pantoea*, respectively. Isolate E3 is most likely *Pantoea agglomerans*.

2.3.1.1 *Acinetobacter*

The genus *Acinetobacter* consist of oxidase negative aerobic gram negative coccobacilli that can be found on human skin and in mucus membranes, water, soil, food, vegetation and sewage (Rahal, Urban, & Ph, 2000). *Acinetobacter* species, along with other nonpathogenic gram-negative bacteria are known to be numerous on food processing surfaces. They are known to exhibit metabolic versatility and can grow at low temperatures and are also able to form biofilms. For example, *Acinetobacter spp*. isolates from a meat abattoir were found to form thick biofilms on polystyrene at 20 ˚C (Giaouris et al., 2015).

Pathogenic bacteria such as *Lm* present in food processing environments can interact with *Acinetobacter* resulting in both inhibitory and stimulatory effects on pathogens in multispecies biofilms (Møretrø & Langsrud, 2017). In a recent study, when *Acinetobacter* was co-cultured with *Lm* and other resident bacteria in the food processing environment (e. g. *Pseudomonas spp*.), the non-*Lm* strains reached the highest cell counts in suspension and in biofilms formed on coupons made of conveyor belt material, but *Lm* in the multigenera biofilm also increased in numbers during the course of the experiment (Fagerlund et al., 2017).

2.3.1.2 *Pedobacter*

Members of the *Pedobacter* genus are gram-negative rod-shaped psychotropic microorganisms. Most *Pedobacter* species are pigmented ranging from yellow to pink. The *Pedobacter* species isolated for this study formed pinkish round colonies typically 1 - 4 mm in diameter on R2A agar after 24 hours of incubation at 25- 30 ˚C. All representatives of this genus have been isolated from environmental, terrestrial and aquatic habitats such as soil, activated sludge and freshwater bodies (Margesin & Shivaji, 2015).

2.3.1.3 *Chryseobacterium*

Members of the *Chryseobacterium* genus are generally part of the environmental flora but may cause infection in humans especially in neonates and immunocompromised, albeit rarely. They are ubiquitous in nature, being found in soil, plants, fresh and marine water and also in chlorine-treated municipal water supplies (Alfouzan et al., 2014). Little is known about virulence factors of *Chryseobacterium* species, but biofilm formation plays a role in their persistence in different environments. A recent study on identification, characterization and biofilm-forming ability of *Chryseobacterium gluem* isolates, observed a mesh-like biofilm structure resembling that of *Lm* (Begley et al., 2009; Lo & Chang, 2014).

2.3.1.4 *Pantoea agglomerans*

Pantoea agglomerans is frequently associated with plant material. It is known to cause damage to plants such as to the cotton seed ball and to cause bacterial blight in some edible mushrooms (Dutkiewicz et al., 2016). Members of this species formed stronger biofilms under various environmental conditions than other gram negative bacteria such as *Serratia* spp. (Naher et al., 2014). Nothing is known about co-culturing *Lm* with *Pantoea agglomerans* but owing to the fact that it was isolated from an environment where *Lm* is likely present, it is likely that the two species interact in the development of a multispecies biofilm structure.

Table 3. Blast results for the 16S rRNA gene sequence fragment from isolate J1 (sequences with the highest identity scores are shown)

Description from BLAST	Max score	Query cover	E value	Identity	Accession
Acinetobacter sp. 19WI (-) OTU39 partial 16S rRNA gene, isolate 19WI (-) OTU39	326/326	100%	1e-85	99%	LN812284.1
Acinetobacter sp. q42 16S ribosomal RNA gene, partial sequence	326/326	100%	1e-85	99%	EU375648.1
Acinetobacter sp. JB19 16S ribosomal RNA gene, partial sequence	326/326	100%	1e-85	99%	EF103567.1
Acinetobacter sp. JB17 16S ribosomal RNA gene, partial sequence	326/326	100%	1e-85	99%	EF103566.1
Acinetobacter sp. JB11 16S ribosomal RNA gene, partial sequence	326/326	100%	1e-85	99%	EF103563.1
Acinetobacter sp. JB5 16S ribosomal RNA gene, partial sequence	326/326	100%	1e-85	99%	EF103560.1
Acinetobacter sp. JB3 16S ribosomal RNA gene, partial sequence	326/326	100%	1e-85	99%	EF103559.1

Table 4. Blast results for the 16S rRNA gene sequence fragment from isolate I5 (sequences with the highest identity scores are shown)

Description from BLAST	Max score	Query cover	E value	Identity	Accession
Uncultured bacterium partial 16S rRNA gene, clone AF- 36956	1085/1085	100%	0.0	99%	LT595872.1
Uncultured bacterium partial 16S rRNA gene, clone AF- 36005	1085/1085	100%	0.0	99%	LT595862.1
Uncultured bacterium partial 16S rRNA gene, clone AF- 35853	1085/1085	100%	0.0	99%	LT595836.1
Uncultured bacterium partial 16S rRNA gene, clone AF- 35478	1085/1085	100%	0.0	99%	LT595793.1
Uncultured bacterium partial 16S rRNA gene, clone AF- 35352	1085/1085	100%	0.0	99%	LT595785.1
Uncultured bacterium partial 16S r RNA gene, clone AF- 35201	1085/1085	100%	0.0	99%	LT595758.1
Pedobacter suwonensis for 16S rRNA, partial sequence, strain: NBRC 106384	1085/1085	100%	0.0	99%	AB682400.1

Table 5. Blast results for the 16S rRNA gene sequence fragment from isolate I3 (sequences with the highest identity scores are shown)

Description from BLAST	Max score	Query cover	E value	Identity	Accession
Uncultured Chryseobacterium sp. clone y-q 16S ribosomal RNA gene, partial sequence	1299/1299	100%	0.0	100%	KJ781298.1
Chryseobacterium sp. NBRC 101330 gene for 16S rRNA, partial sequence	1299/1299	100%	0.0	100%	AB681455.1
Chryseobacterium sp. M229 gene for 16S rRNA, partial sequence, strain: M229	1299/1299	100%	0.0	100%	AB461706.1
Uncultured bacterium clone SZ201108-29 16S ribosomal RNA gene, partial sequence	1269/1269	100%	0.0	99%	KX508685.1
Chryseobacterium sp. FSBSY15 16Sribosomal RNA gene, partial sequence	1266/1266	100%	0.0	99%	KJ184983.1

Table 6. Blast results for the 16S rRNA gene sequence fragment from isolate E3 (sequences with the highest identity scores are shown)

Description from BLAST	Max score	Query cover	E value	Identity	Accession
Pantoea agglomerans strain DsppB7 16S ribosomal RNA gene, partial sequence	1125/1125	100%	0.0	100%	KT958491.1
Pantoea agglomerans strain CZ-BHG003 16S ribosomal RNA gene, partial sequence	1125/1125	100%	0.0	100%	KT765839.1
Bacterium DS4EC-385 16S ribosomal RNA gene, partial sequence	1125/1125	100%	0.0	100%	KU726462.1
Pantoea agglomerans strain FDAARGOS 160, complete genome	1125/7813	100%	0.0	100%	CP014129.1
Pantoea agglomerans strain TAbd1 16S ribosomal RNA gene, partial sequence	1125/1125	100%	0.0	100%	KT184494.1

2.3.2 Biofilm forming ability of the six *Lm* strains and four PPPI

Table 7. Relative biofilm formation by Lm and produce processing plant isolates (PPPI) and their mixtures as determined by crystal violet staining optical density (OD) at 600 nm ± SD after 5 days biofilm growth at 22 ^{0}C. Different letters (A, B and C) denote mean OD values that are statistically significant (P<0.05).

Strain	Average OD @ 600 nm	Biofilm
***Lm* 390**	0.083 ± 0.021	Weak- Moderate
***Lm* Scott A**	0.112 ± 0.059	Moderate
***Lm* 107**	0.086 ± 0.047	Weak- Moderate
***Lm* 108**	0.060 ± 0.036	Weak
***Lm* 506**	0.063 ± 0.045	Weak
***Lm* 110211**	0.100 ± 0.074	Moderate
J1	0.945 ± 0.448	Strong
I5	0.096 ± 0.034	Moderate
I3	0.110 ± 0.094	Moderate
E3	0.212 ± 0.032	Strong
***Lm* + PPPI (J1, I5, I3 &E3)**	0.187 ± 0.089 **C**	Moderate- Strong
***Lm* cocktail**	0.084 ± 0.047 **B**	Weak- Moderate
Control	0.036 ± 0.002 **A**	No biofilm

Lm strains 108 and 506 presented as weak biofilm formers, while stains *Lm* 390 and *Lm* 107 demonstrated weak to moderate biofilm formation, and strains *Lm* Scott A and *Lm* 110211 were moderate biofilm formers. The PPPI strains I5 and I3 were

moderate biofilm formers while J1 and E3 demonstrated strong biofilm-forming ability. The multispecies biofilm formed by combining the six *Lm* strains and the four PPPI's was moderate to strong and sufficient to work with for subsequent experiments. It has been previously shown that microorganisms including *Lm* adhere in higher numbers to hydrophobic material such as the polystyrene plates used in this assay. As adhesion is the first step in complex process of biofilm formation, this could be one possible explanation for the ability of the *Lm* strains in conjunction with other four PPPI's to produce a moderately strong biofilm on the polystyrene microtiter plates used (Pompermayer & Gaylarde, 2000).

The greater biofilm formation achieved by PPPI strains J1 and E3 compared to the *Lm* strains agrees with the published superiority of gram negative bacteria to form biofilms on inert surfaces (Takeuchi et al., 1999). Bacteria in a food processing environment may be exposed to different levels of nutrients, depending upon the location in a plant. However, it is well known that many factors influence biofilm formation, among them the composition of the medium. Rich nutrient medium has been known to cause greater *Lm* biofilm growth (Hood & Zottola, 1997a). Nevertheless, R2B, a very low nutrient medium, was chosen in this investigation to mimic produce processing industry conditions which might not provide as much nutrition as rich laboratory media such as TSBYE (Leriche & Carpentier, 2000).

In the past, most of the research on biofilms focused on single species biofilms; however, biofilms in nature mostly consist of multiple species where interactions between species shape the development, structure and function of these microbial communities. Therefore, in recent years, a gradual shift in focus towards examining the complexity and interactions of multispecies biofilms has occurred (Yang et al., 2011). Such interactions as seen with the *Lm* and PPPI bacteria used for this study have been found to influence growth, survival and potential virulence properties of the bacteria which in turn influence overall pathogenicity. Also, it is well accepted that bacteria in biofilms are more protected against various stresses than planktonic exponentially growing cells. Several studies have confirmed that interactions between different bacteria in a biofilm could potentially influence their relative resistance to antimicrobial agents such as disinfectants (Bridier et al., 2011; Burmølle et al., 2014; Elias & Banin, 2012; Fagerlund et al., 2017).

Mutagenesis approaches have revealed that flagella and their motility play a role in *Lm* biofilm formation (Chang et al., 2012). However, this role seems to be limited to the positive role afforded by motility on the initial steps of surface attachment, probably by increasing the likelihood of encountering a surface and overcoming the repelling electrostatic forces and not by flagella necessarily acting as surface adhesins (Jaglic et al., 2018). Furthermore, contrary to the extracellular matrices of many microbial biofilms, *Lm* has been reported to lack exopolysaccharides. Electron microscopy has only sometimes revealed the presence of putative fibril-

like structures binding cells to each other or to the surface (Giaouris et al., 2015). This apparent lack of extracellular polymeric substances could be the explanation of the observed low OD values resulting in weak to moderate single strain *Lm* biofilms observed in the present study.

Some studies have found increased attachment of *Lm* to wet surfaces pre- colonized with non-pathogenic bacteria (*Pseudomonas spp.* and *Acinetobacter spp.)* that are resident in most food processing surfaces and that survive cleaning and disinfection (Chmielewski & Frank, 2003; Hassan et al., 2004). The change in surface properties due to resident bacteria EPS production which enhanced attachment of *Lm* was the most likely explanation for increased biofilm production when the six *Lm* strains were co-cultured with the four PPPI's. In another relevant study of 29 gram-negative and gram-positive isolates from food processing plants, 13 percent of the strains increased *Lm* CFU counts in the biofilms, while the others had a negative or no effect on *Lm* populations (Carpentier & Chassaing, 2004).

Studies done with *Lm* in biofilms also harboring *Pseudomonas* and *Acinetobacter spp*. have shown that *Lm'*s tolerance to sanitizers and disinfectants is higher than that in *Lm*- only biofilms (Fagerlund et al., 2017; Van der Veen & Abee, 2011). However, the protective effects of multiple species within the biofilm have been difficult to judge given the complexity of the multispecies biofilm structures, and much remains to be investigated.

Chapter 3

MINIMUM BIOFILM ERADICATION CONCENTRATION (MBEC)

3.1 Introduction

The MBEC™ (minimum biofilm eradication concentration) assay is used to determine the efficacy of an antimicrobial agent in inactivating biofilm-associated cells. The assay uses a Calgary Biofilm Device (CBD), a 96-well plate with pegs built into the lid that allow for the adherence and growth of biofilm. MBEC assays are very time efficient and an accurate method of testing antimicrobial agent efficacy against biofilm cells because they allow carrying out 96 simultaneous tests.

Usually, a biofilm is allowed to form on the pegs as illustrated in **Figure 3**. Loosely attached cells can be removed from the pegs simply by dipping them into a rinsing solution such as phosphate-buffered saline before placing the pegs into a microtiter plate containing desired concentrations of antimicrobial agents. After the desired treatment time, the pegs are removed from the microtiter plate and can either be placed into fresh media to observe survivors of the treatment or the pegs can be broken off the lid and after removal of the cells from the pegs by sonication, the number of surviving cells can be determined by plating on appropriate media. The MBEC is the lowest concentration of the antimicrobial

that rids the peg of all culturable cells, indicated by the absence of growth when the pegs are transferred to suitable growth media. Alternatively, a spectrophotometer can be used to find the optical density (OD) of the content of the wells.

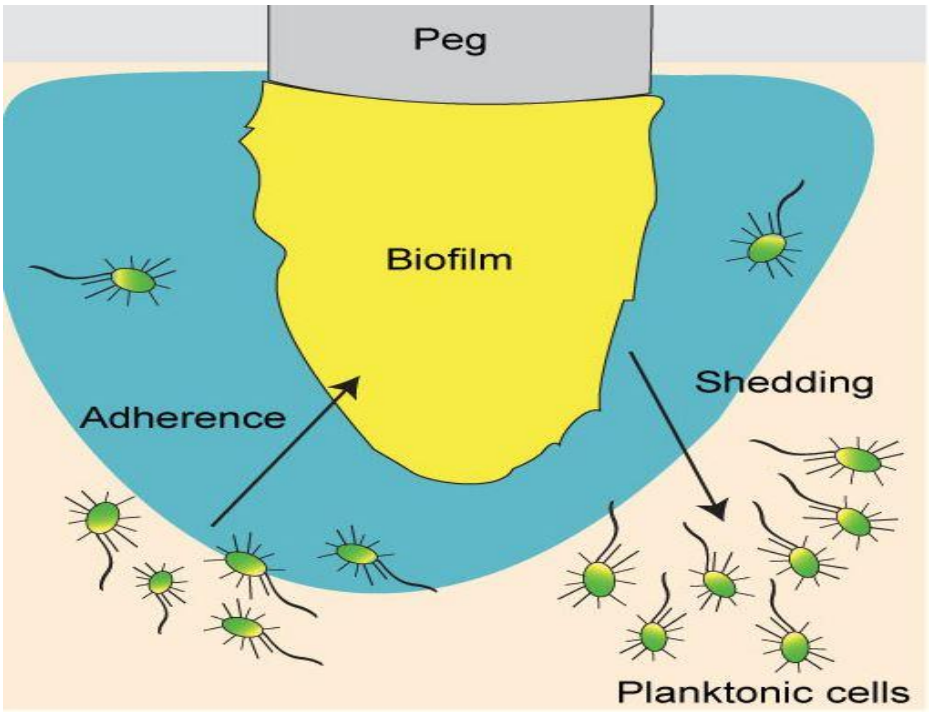


Figure 3. Illustration of a mature biofilm in a peg of the CBD system.

3.2 Materials and Methods

3.2.1 Biofilm growth in microtiter plate wells and on pegs of Calgary Biofilm Device (CBD)

Biofilms were grown as described in **section 2.2.3** with a few modifications. After adjusting the OD to 0.05 for each strain grown in R2B overnight, equal volumes of each *Lm* strain were combined and 150-µl aliquots were added to microtiter plate wells for the development of pure *Lm* biofilms. For growth of mixed-species biofilms, equal volumes of OD-adjusted cultures of each of the four produce processing plant isolates were combined and the resulting mixture was combined with the *Lm* strain mixture at a volume ration of 1:1. Aliquots of 150 µl were pipetted into microtiter plate wells. For biofilm growth on the pegs of a Calgary biofilm device, the pegged lid of the device was used to cover the plate. The assembly was incubated at 22 ^{0}C to allow cell attachment and after 4 hours, the suspension was removed and replaced with fresh R2B medium. The cells adhering to the pegs or microtiter surface were allowed to form biofilms over a period of 5 days with media replacement every 24 hours.

3.2.2 Determination of the best method of dislodging *Lm* cells from the biofilm prior to antimicrobial treatment.

Biofilms were grown on flat bottom wells (24 well plates) as described in **section 2.2.3**. The R2B medium suspension was removed and the wells were washed thrice with PBS to remove loosely attached cells. One ml of sterile tap water was added to each well and the plates were either sonicated for 30 s or shaken for 15 minutes on a Corning LSE Digital Microplate shaker model S2020-P4-COR) with rolling glass beads at a setting of 30 rpm or 15 minutes shaking without beads followed by 5 min of sonication. Serial dilutions of the well contents were done in PBS and 100 µl of the dilutions were plated in triplicate on Brilliance Listeria Agar (BLA). The plates were incubated for 24-48 hours at 37^0C. Green colonies present were counted and expressed as CFU/ ml tap water.

3.2.3 Determination of the minimum biofilm eradication concentration (MBEC)

Sodium hypochlorite solutions were prepared by dilution of commercial bleach in autoclaved tap water. The pH was checked and adjusted to the range 6.5- 7.5 with 10% hydrochloric acid (HCl). The commercially available peroxyacetic acid/

hydrogen peroxide solutions, Vigor Ox 15 F&V (15% PAA and 10% H_2O_2) was diluted to appropriate concentrations in autoclaved tap water.

Pegs with biofilms were dipped once in PBS to remove unattached cells and then lowered into 200 µl of sodium hypochlorite (0-2000 ppm) or peroxyacetic acid/ hydrogen peroxide (0- 960 ppm) solutions. The CBD assembly was placed on a Corning LSE Digital Microplate shaker model S2020-P4-COR) for 7.5, 15 and 30 minutes at a speed of 30 rpm. The CBD pegs were dipped in Difco™ D/E Neutralizing Broth (NB) for 1 minute, transferred into wells containing 200 µl TSBYE and incubated for 24 hours at 37˚C. Aliquots of the TSBYE cultures were spotted onto *Listeria*-selective Brilliance Listeria Agar (BLA). The plates were incubated for 24 – 48 h at 37˚C and the appearance of green colonies indicated the presence of *Lm*.

3.3 Results and Discussion

3.3.1 Dislodging of *Lm* cells prior to antimicrobial treatment

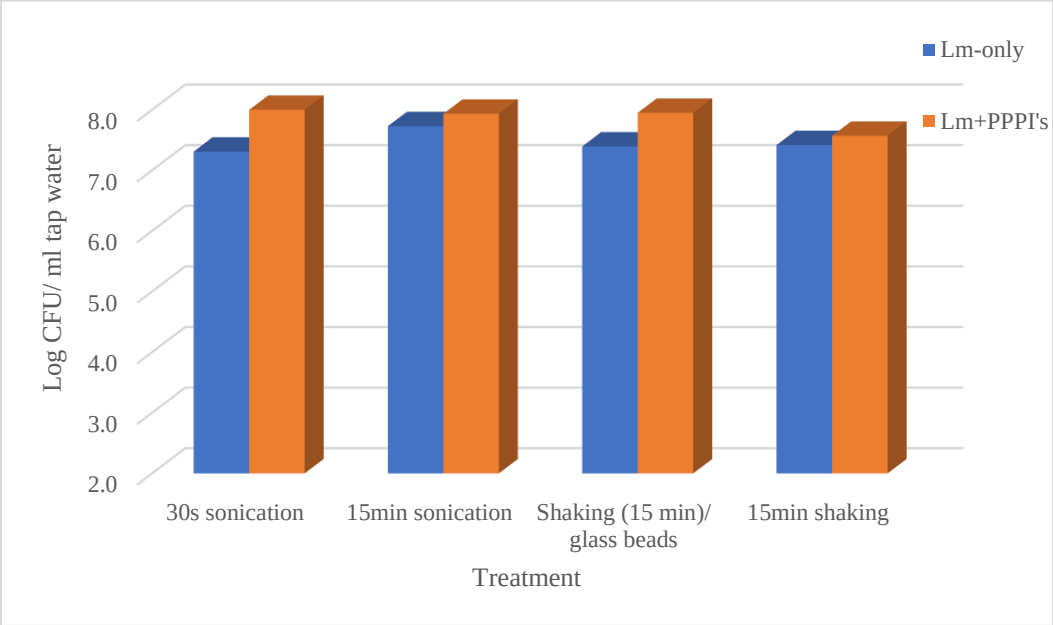


Figure 4. Log CFU of Lm cells released from Lm-only and mixed-species biofilms grown on pegs of the CBD by different mechanical treatments. Values are the average of two replicates.

Fig. 4 shows $\log_{10}$ CFU of Lm cells released from one peg into 1 ml tap water obtained after mechanical treatments; 30s or 15 min sonication and 15 min shaking with and without glass beads of the biofilm wells. This test was performed to help determine the most effective way of dislodging *Lm* cells in pure and multispecies biofilms prior to antimicrobial treatments. It was observed that all the techniques applied yielded CFUs in the log 7- 8 range. However, higher values were obtained

with multispecies biofilms (*Lm* + PPPI) than with *Lm*-only biofilms across all mechanical treatments. This finding is an indication that irrespective of the mechanical manipulation applied to the biofilm prior to the antimicrobial treatment, the number of *Lm* cells coming off the biofilm remains fairly constant. This could owe to the fact that *Lm* does not form very strong biofilms as demonstrated in previous studies. This means that the cells easily come off.

In an experiment to evaluate dislodging methods for *Pseudomonas fluorescens* and *Bacillus subtilis* biofilms grown on stainless steel or polyurethane surfaces, Lindsay & Holy (1997) found no significant differences ($P > 0.05$) between bacterial counts resulting from vortexing, sonication or shaking with beads However, scanning electron microscopy suggested that shaking with beads removed cells as well as residues of extra polymeric substances (EPS) more efficiently from both surfaces for both bacteria. Therefore, shaking with beads (where applicable) was used to remove *Lm* cells in the present experiments.

3.3.2 Minimum *Lm* biofilm eradication concentration (MBEC).

3.3.2.1 Susceptibility to sodium hypochlorite (NaOCl)

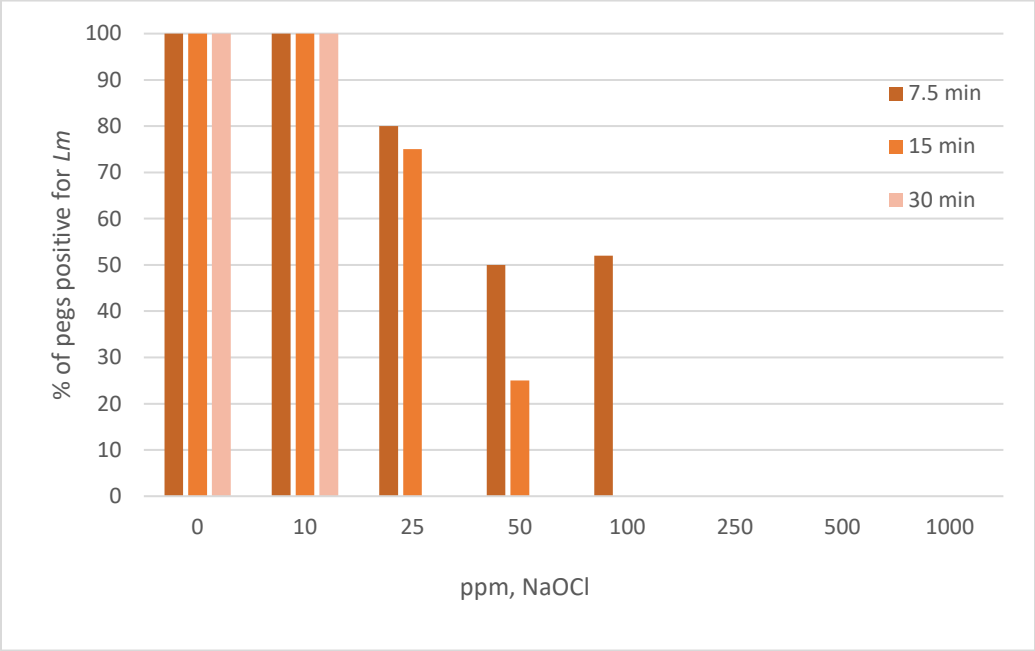


Figure 5. Percent of pegs with multispecies biofilms (Lm + PPPI) that tested positive for* Lm *following treatments with initial concentrations of 0 - 1000 ppm NaOCl in tap water for 7.5, 15 and 30 minutes at room temperature. Data are from for replicate pegs.

Complete inactivation of *Lm* in the multispecies biofilm required a 30-min exposure to 25 ppm NaOCl in tap water at pH 6.8 or a 7.5-min exposure to 50 ppm NaOCl in tap water at the same pH. These times and concentrations are markedly higher than those required for inactivation of planktonic *Lm* cells (Brackett, 1987; Hood & Zottola, 1997b).

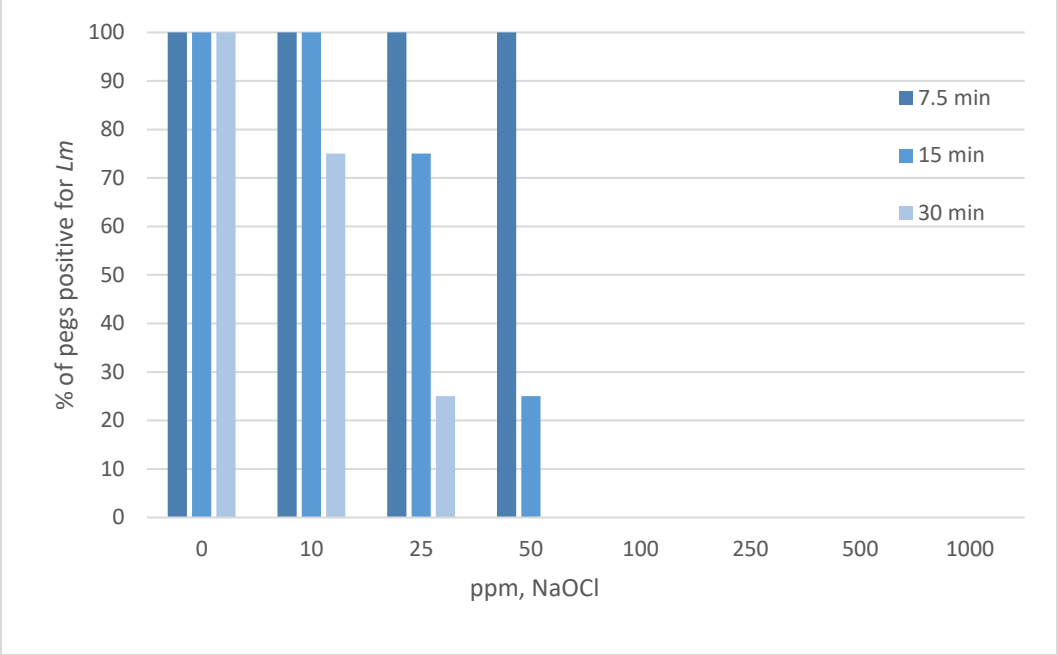


Figure 6. Percent of pegs with* Lm*-only biofilms that tested positive for* Lm *following treatments with 0 - 1000 ppm NaOCl in tap water for 7.5, 15 and 30 minutes at room temperature. Data for four replicate pegs.

The data indicates that *Lm* cells from pure biofilms were approximately two-fold more sensitive to the antimicrobial than *Lm* in the multispecies biofilms. Here, 30-min exposures at a concentration of 50 ppm or 7.5-min exposures to 100 ppm NaOCl were sufficient to completely inactivate *Lm* cells from the *Lm*-only biofilms.

3.3.2.2 Susceptibility to Peroxyacetic acid (PAA)

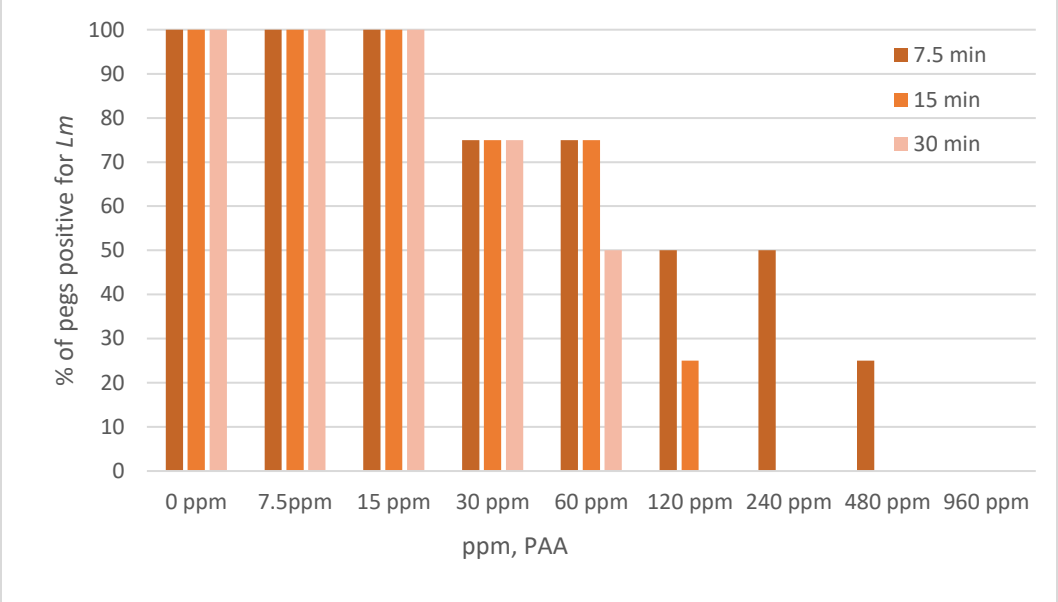


Figure 7. Percent of pegs with multispecies biofilms (*Lm *+ PPPI) that tested positive for* Lm *following treatments with 0- 960 ppm PAA in tap water for 7.5, 15 and 30 minutes at room temperature. Data are for four replicates.

Fig. 7 shows the effect of PAA on multispecies biofilms grown and treated at room temperature. Similar to NaOCl treatments, a slightly higher concentration and exposure time was required for complete inactivation of *Lm* in the multispecies biofilm (240 ppm and 120 ppm for 7.5 and 30 minutes, respectively) than in pure *Lm* biofilm (120 ppm and 15 ppm for 7.5 and 30 minutes, respectively).

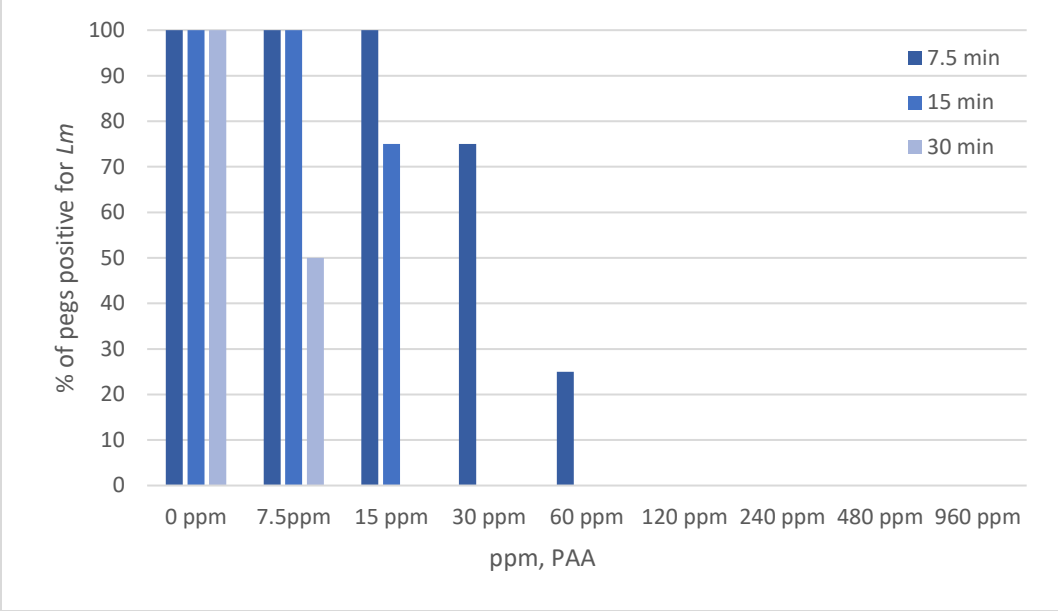
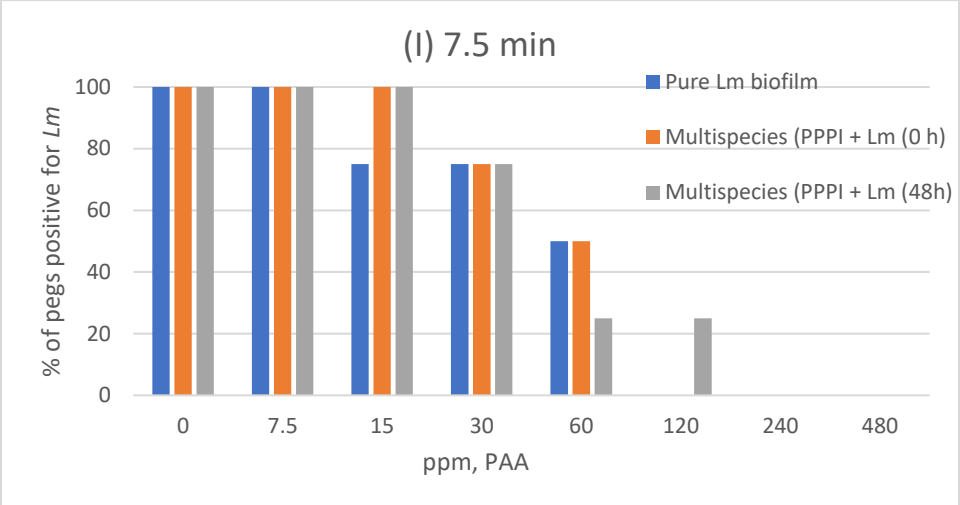


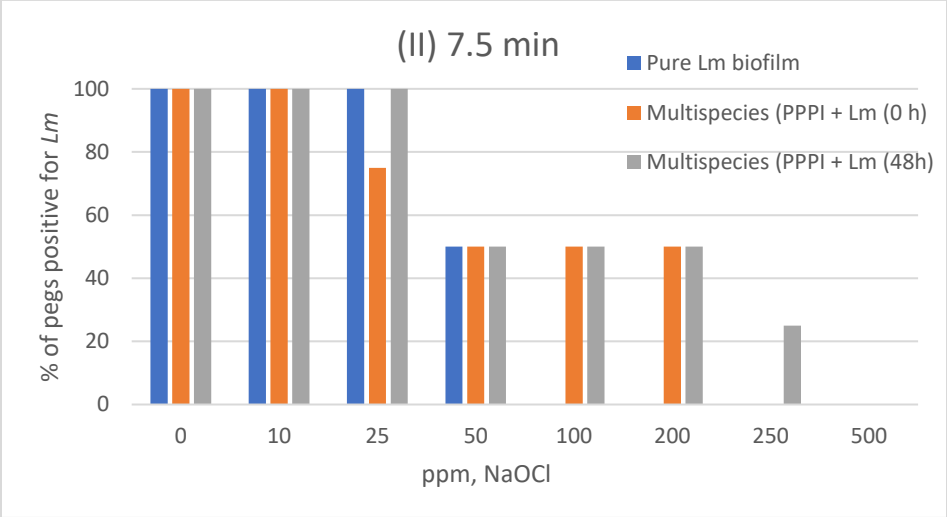
Figure 8. Percent of pegs with* Lm-*only biofilms that tested positive for* Lm *following treatments with 0- 960 ppm PAA in tap water for 7.5, 15 and 30 minutes at room temperature. Data are for four replicates.

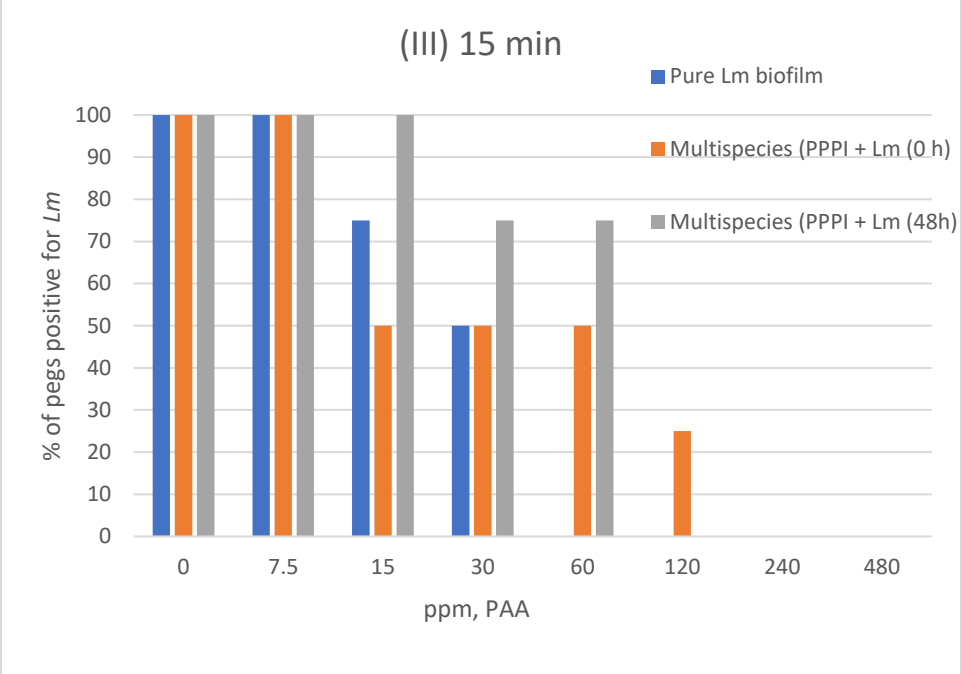
3.3.2.3 Susceptibility of *Lm* added to existing biofilm to NaOCl and PAA

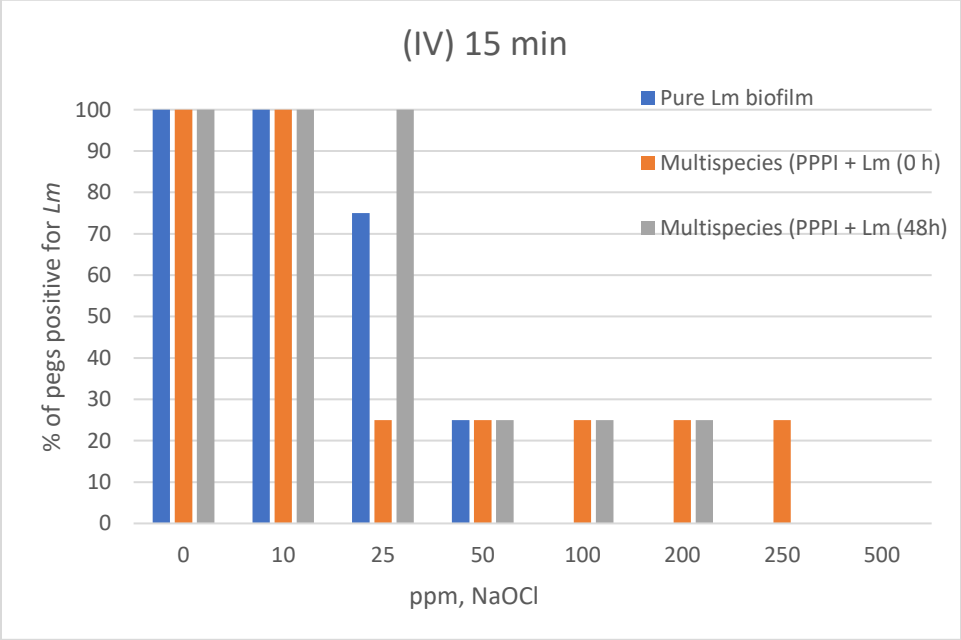
This assay was done to mimic a situation in which planktonic *Lm* cells encounter and interact with an existing biofilm at room temperature. As described in section 3.2.1, the PPPI strains were allowed to form biofilms for 48 hours after which their medium (R2B) was replaced with a suspension of the six *Lm* strains. After 4 h, the suspension was replaced with fresh R2B medium and additional media replacements were done every 24 h for 3 days. Pure *Lm* and mixed biofilm at onset

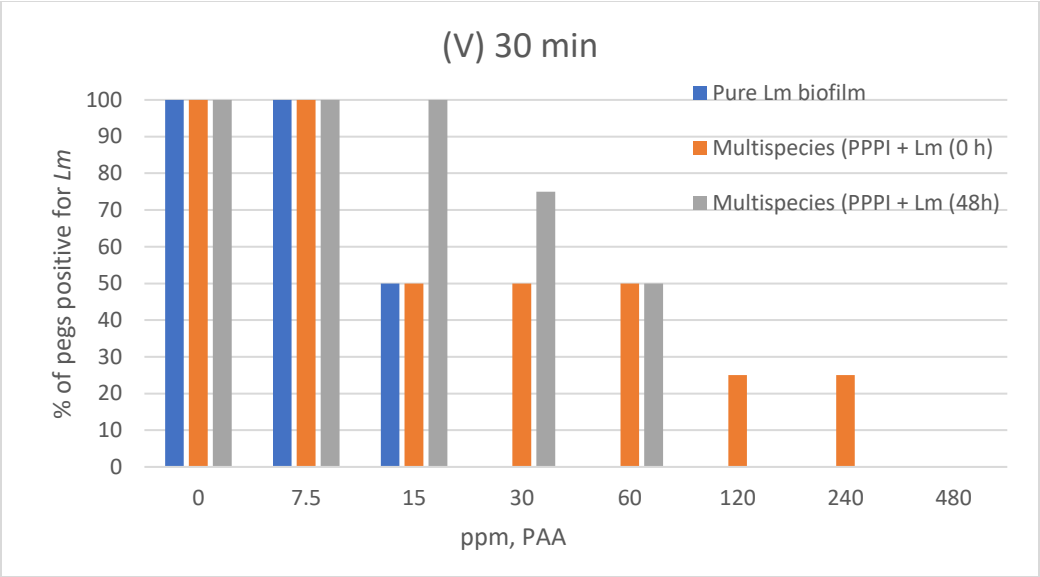
were used as the comparison group. The Figures below show side-by-side comparison of the effects of varying concentrations of NaOCl or PAA and exposure times on *Lm* added to existing biofilm at room temperature.






(III) 15 min
Pure Lm biofilm
Multispecies (PPPI + Lm (0 h)
Multispecies (PPPI + Lm (48h)
% of pegs positive for Lm
100
90
80
70
60
50
40
30
20
10
0
0
7.5
15
30
60
120
240
480
ppm, PAA


(IV) 15 min
Pure Lm biofilm
Multispecies (PPPI + Lm (0 h)
Multispecies (PPPI + Lm (48h)
% of pegs positive for Lm
100
90
80
70
60
50
40
30
20
10
0
0
10
25
50
100
200
250
500
ppm, NaOCl



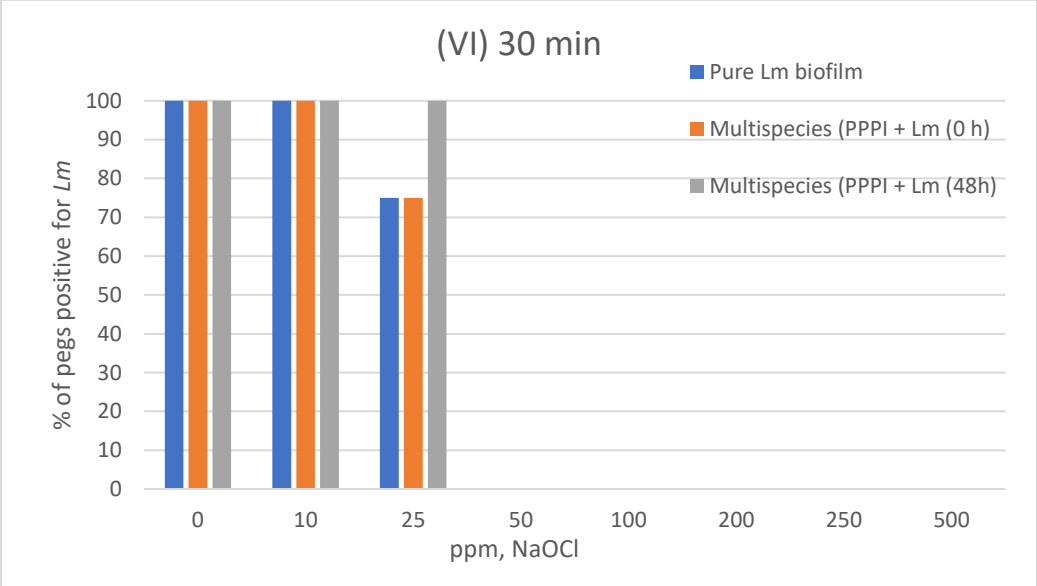


Figure 9. Percent of pegs in CBDs positive for* Lm *after treatment with different concentrations of PAA or NaOCl for 7.5 (I &II), 15 (III &IV), or 30 min (V &VI). The blue bars indicate results for peg-associated pure* Lm *biofilms, the orange bars are for mixed species, room temperature-grown biofilms and the gray bars

represent results for mixed-species biofilms produced by adding* Lm *to a 2-day-old biofilm of the four produce processing plant isolates from four independent trials.

The above Figures show that *Lm* added to existing biofilms (PPPI + *Lm* (48 h) was about as sensitive to the antimicrobial as *Lm* that was part of the biofilm from the start (PPPI + *Lm* (0 h) across all time points assayed.

Chapter 4

EFFICACY OF SODIUM HYPOCHLORITE OR PEROXYACETIC ACID TO PREVENT TRANSFER OF *LISTERIA MONOCYTOGENES* FROM BIOFILMS TO CBD PEGS AND SPINACH

4.1 Introduction

The introduction of *Lm* into processing plants is likely unavoidable given the ubiquitous presence of this organism in the environment. In a suspended state, such Lm cells are likely transient, but once able to attach to a surface or settle in crevices or cracks, the bacterium may persist in the plant for decades. Persistence on food contact surfaces is of great concern because the presence of *Lm* increases the risk for product contamination (Hood & Zottola, 1997b). Several factors are involved in the transfer of *Lm* to foods such as surface type, nature of biofilm, hydration level and food type. In his study, Rodriguez et al. (2007) found that transfer of *Lm* to bologna and American cheese was more efficient from stainless steel than from high-density polyethylene surfaces and higher transfer of *Lm* from surfaces that had biofilm than from surfaces that had planktonic cells (Rodríguez & McLandsborough, 2007).

Treatment of food contact surfaces with antimicrobial agents to prevent the transfer of *Lm* to food is a critical point for food safety assessment (Lee et al., 2014). Past studies have looked at the use of antimicrobial agents to inactivate *Lm* deposited and dried on a surface. . For instance, in one study, 200 ppm sodium hypochlorite was capable of reducing *Lm* from Brussel sprouts with an initial *Lm* load of ~6 Log_{10} CFU/g by 2 Log_{10} CFU/g in 20 seconds while tap water alone only reduced this load by about 1 Log_{10} CFU/g (Brackett, 1987). It was not tested if the cells remaining on the Brussel sprouts were able to be transferred to other items. Overall, there is insufficient research on transfer of *Lm* from biofilms to other surfaces or fresh produce. This study focused on the efficacy of sodium hypochlorite and peroxyacetic acid to prevent transfer of *Lm* from existing biofilms on stainless steel to CBD pegs and spinach.

4.2 Materials and Methods

4.2.1 Test for prevention of transfer of *Lm* from a biofilm to sterile surfaces (pegs)

Biofilms were grown as described in **section 3.2.1**. The wells were washed thrice with PBS to remove unbound cells and 200 µl of sodium hypochlorite or PAA solutions were added. Sterile Calgary pegs were lowered into the microtiter plate wells and the assembly was kept for 7.5 and 15 minutes on a Corning LSE Digital

Microplate shaker model S2020-P4-COR. The pegs were dipped in neutralizing broth (NB) for 1 min followed by incubation in Listeria Enrichment Broth (LEB) for 24 hours at 37 ˚C. Aliquots of the LEB culture medium were spotted onto *Listeria*-selective Brilliance Listeria Agar (BLA). The plates were incubated 24 – 48 h at 37 ˚C and the appearance of green colonies indicated the presence of *Lm*.

4.2.2 Test for prevention of transfer of *Lm* from a biofilm to surfaces colonized by other bacteria

Pure *Lm* strains biofilms were developed in a 96-well microtiter plate and produce processing plant isolates biofilms were developed on CBD pegs as described in **section 3.2.1**. The wells/pegs containing the biofilm were washed once with PBS to remove unbound cells. Two-hundred µl of sodium hypochlorite or PAA solutions were added to wells containing *Lm*- biofilm and pegs coated with the PPPI strains were lowered into the wells. The assembly was placed on a Corning LSE Digital Microplate shaker model S2020-P4-COR for 7.5 and 15 minutes. The pegs were dipped in neutralizing broth for 1 minute followed by incubation in Listeria Enrichment Broth (LEB) for 24 hours at 37 ˚C. Aliquots of the LEB culture medium were spotted onto *Listeria*-selective Brilliance Listeria Agar (BLA). The plates were incubated 24 – 48 h at 37 ^{0}C and the appearance of green colonies indicated the presence of *Lm*.

4.2.3 Test for prevention of transfer of *Lm* from a biofilm to fresh spinach

Mixed species biofilms were developed on stainless coupons as described in **section 2.2.3** with a few modifications. After adjusting the OD for each strain to 0.05 (both *Lm* and PPPIs) grown in R2B overnight, equal volumes were combined at a ratio of 1:1 to form a mixed strain cocktail. Aliquots of 100 µl were pipetted into stainless coupons placed in 6-well tissue culture plates. The coupons were incubated at 22 ˚C to allow cell attachment and after 4 hours, the suspension was removed and replaced with fresh R2B medium. The cells adhering to the stainless coupon surfaces were allowed to form biofilms over a period of 5 days with media replacement every 24 hours.

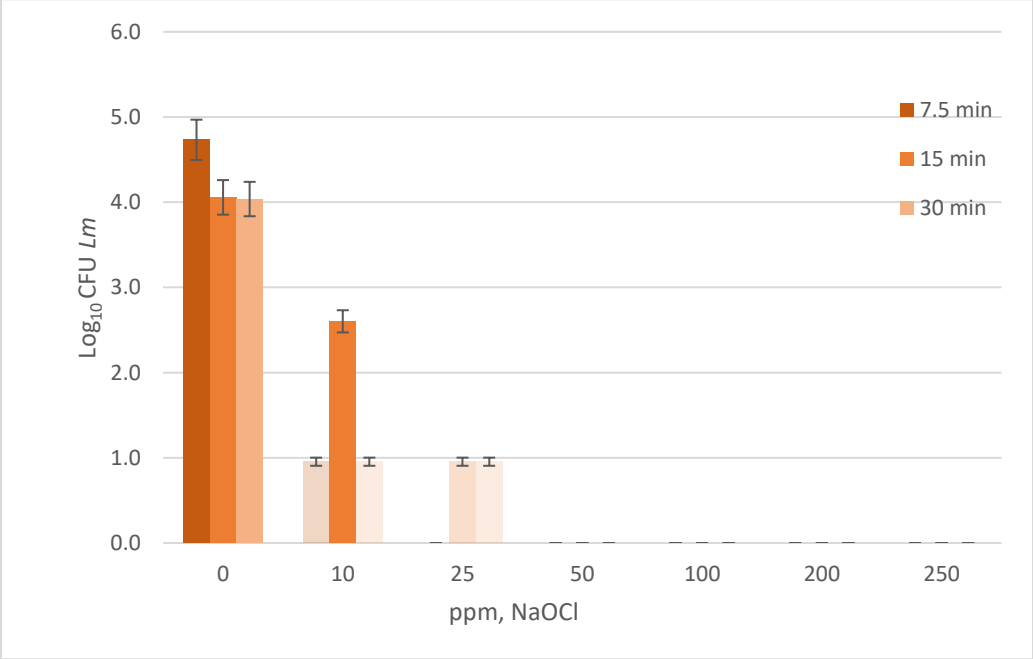
The coupons were washed once by dipping in PBS to remove loosely attached cells. Two coupons were placed at the bottom of a beaker and 200 ml sterile tap water was added. The liquid was stirred with a magnetic stir bar. After 1 min, a 500-µl sample was withdrawn and added to 1 ml of neutralizing broth to determine the number of cells released from the surfaces. Water or antimicrobial solutions were added to obtain final concentrations of 0, 20, 100 and 250 ppm of NaOCl and nine spinach leaves were submerged in the stirred solution.

After 5, 10 and 20 min, three leaves were removed and placed in 50-ml tubes with 10 ml of Neutralizing broth. The contents of the tubes were shaken to expose all leave surfaces to the neutralizing solution. Also, 500-µl of wash solution was

removed at each time point and added to 1 ml of NB. Finally, the coupons were collected, submerged in 3 ml of NB and sonicated to release remaining attached cells. The collected samples were diluted appropriately to obtain counts and samples for which no counts were obtained were subjected to enrichment with LEB.

4.3 Results and Discussion

4.3.1 Transfer in sterile surfaces (pegs of CBD system)



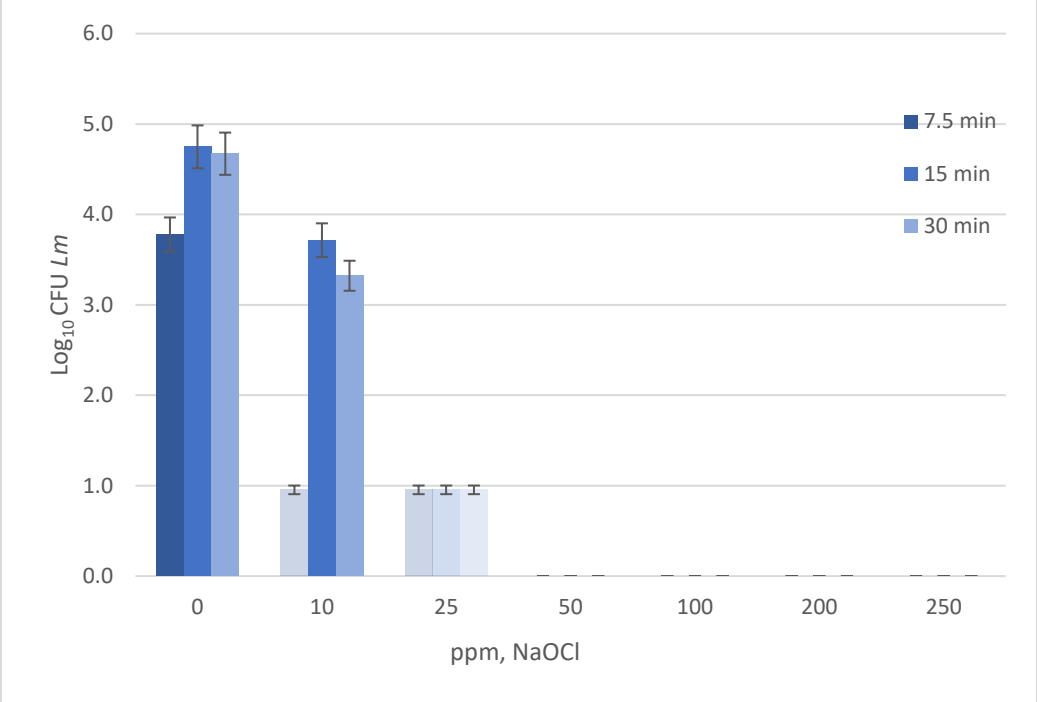
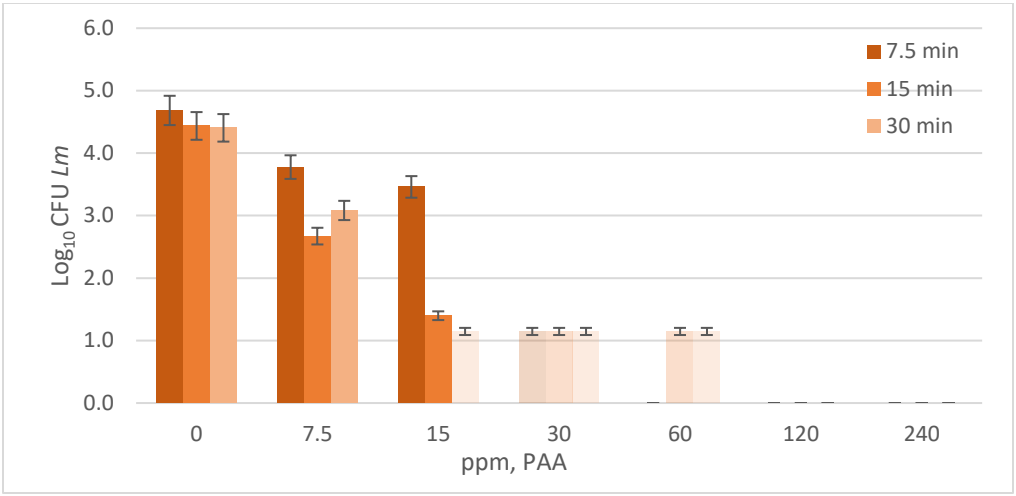


Figure 10. **Lm** ***transfer from the surfaces of microtiter plate wells to sterile pegs in the CBD system in the presence of 0 - 250 ppm NaOCl, done at room temperature. Blue bars show transfer from pure*** **Lm** ***biofilms; orange bars show transfer from multispecies (*** **Lm** ***+ PPPI) biofilm. Translucent bars indicate the detection limit for this assay.***



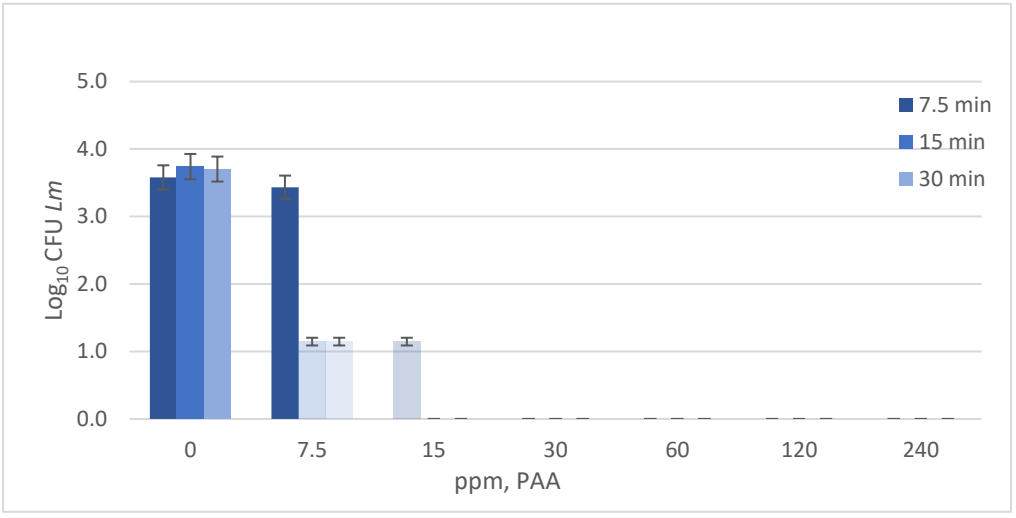


Figure 11. **Lm** ***transfer from the surfaces of microtiter plate wells to sterile pegs in the CBD system in the presence of 0 - 240 ppm PAA, done at room temperature. Blue bars show transfer from pure*** **Lm** ***biofilms; orange bars show transfer from multispecies (*** **Lm** ***+ PPPI) biofilm. Translucent bars indicate the detection limit for this assay.***

4.3.2 Transfer to biofilm-coated pegs of CBD system

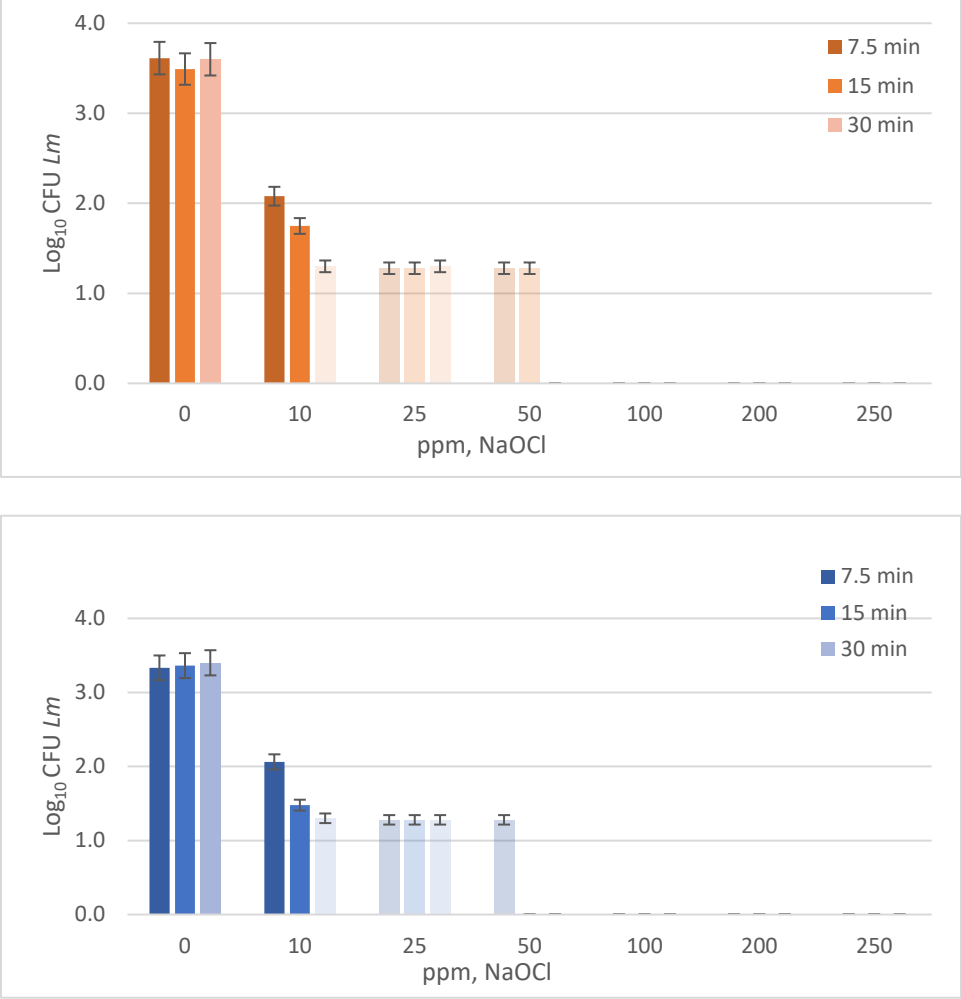


Figure 12. **Lm** ***transfer from the surfaces of microtiter plate wells to non-sterile pegs (coated in PPPI biofilm) in the CBD system in the presence of 0 - 250 ppm NaOCl, done at room temperature. Blue bars show transfer from pure*** **Lm** ***biofilms; orange bars show transfer from multispecies (*****Lm*** ***+ PPPI) biofilm. Translucent bars indicate the detection limit for this assay.***

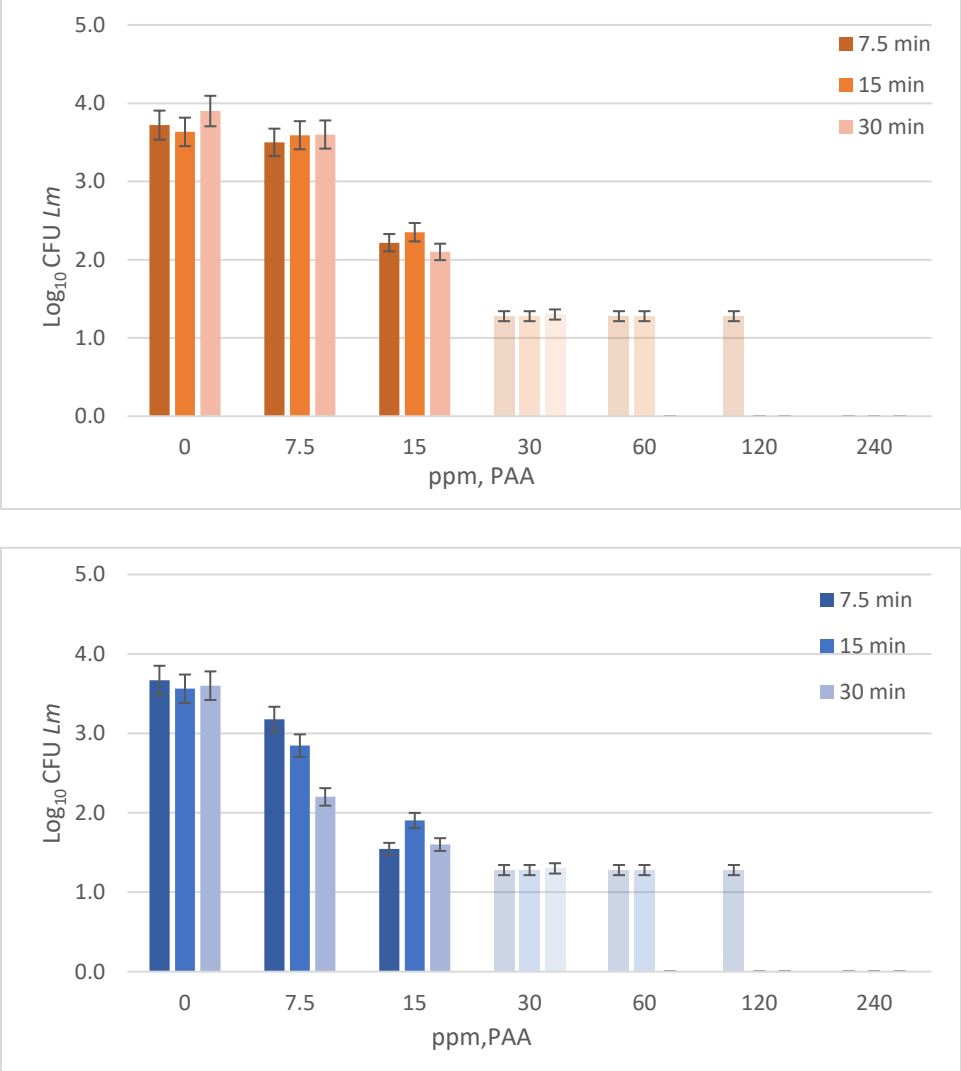


Figure 13. **Lm** ***transfer from the surfaces of microtiter plate wells to non-sterile pegs (coated in PPPI biofilm) in the CBD system in the presence of 0 - 240 ppm PAA, done at room temperature. Blue bars show transfer from pure*** **Lm** ***biofilms; orange bars show transfer from multispecies (*****Lm*** ***+ PPPI) biofilm. Translucent bars indicate the detection limit for this assay.***

Table 8. Minimum concentrations[1] of antimicrobials needed to prevent transfer of* Lm *from pure and mixed- species biofilms to pegs (sterile and biofilm-coated) of the CBD during exposure times 7.5, 15 and 30 minutes.

Antimicrobial agent	**Biofilm type**	**Exposure time**	**Minimum concentrations needed to prevent transfer from biofilms to**	
			Sterile CBD pegs	CBD pegs coated with PPPI
NaOCl	Pure *Lm* species	7.5 min	25	100
		15 min	25	50
		30 min	25	50
	Multispecies (*Lm* + PPPI)	7.5 min	50	100
		15 min	25-50	100
		30 min	25	50
PAA	Pure *Lm* species	7.5 min	15	240
		15 min	7.5	120
		30 min	7.5	60
	Multispecies (*Lm* + PPPI)	7.5 min	30-60	240
		15 min	60-120	120
		30 min	60	60

[1] Minimum concentration is defined as the concentration at which three replicates pegs did not show the presence of Lm after growth in LEB medium and thereafter plating on BLA.

Table 8 shows that transfer of *Lm* from the biofilms to sterile and biofilm-coated pegs was prevented by NaOCl at concentrations between 25 and 50 ppm and 50 and 100 ppm respectively. The concentration of PAA required to prevent transfer was between 7.5 and 120 ppm and 60 and 240 ppm for the two types of surfaces, respectively. These data indicated that biofilm-coated surfaces enable *Lm* released from biofilms to attach and survive the presence of antimicrobials at higher rates than cell-free surfaces. This finding highlights the importance of resident bacteria in the produce processing industry in making it more difficult to prevent transfer of pathogens to new surfaces. This observation also highlights the importance of a good cleaning system prior to performance of sanitation and disinfection processes in the processing area.

Surface microbiota in form of biofilms may offer some level of protection to *Lm* against disinfectants once they were able to attach or penetrate these biofilms, thus higher concentrations of antimicrobials will be required to achieve complete prevention of transfer and survival of *Lm*. It depends on the regulatory, economic and technical circumstances in the processing plant and the type of produce being handled whether or not increased antimicrobial concentrations can be applied.

Nonetheless, it can be observed that under sterile (clean) conditions, it is possible to prevent transfer and survival of *Lm* from existing biofilms at concentration less or equal to the ones generally in use by produce processors.

4.3.3 Transfer of *Lm* from multispecies biofilm on stainless steel coupons to spinach in the presence on NaOCl

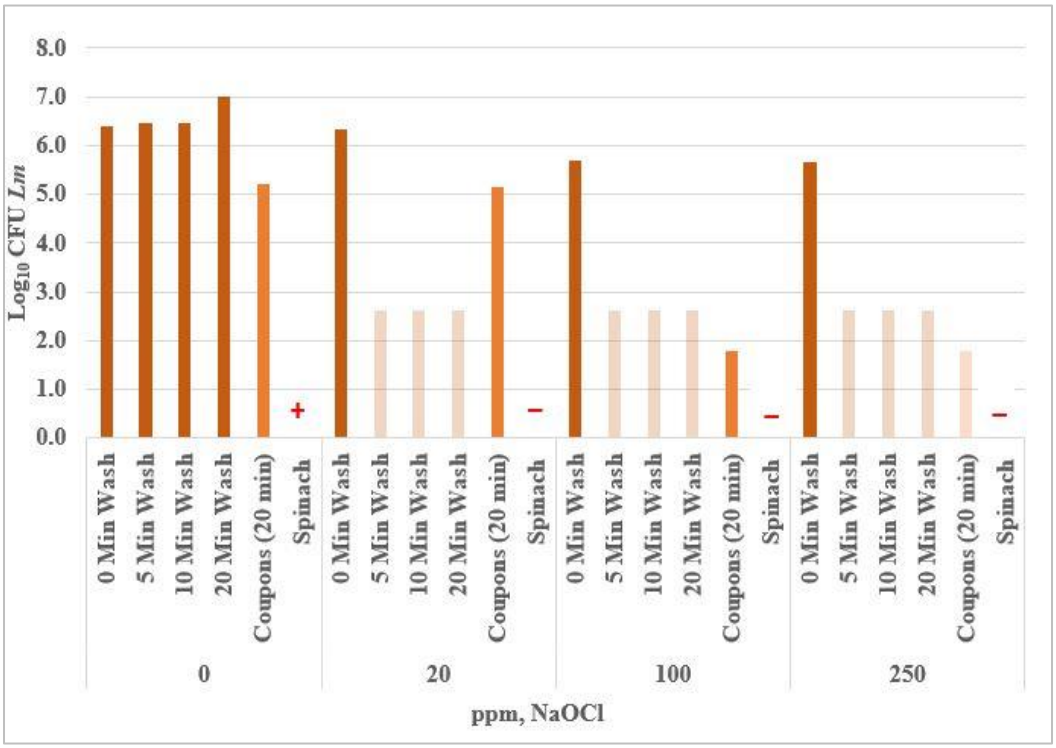


Figure 14. Log_{10} CFU* Lm *counts in wash water containing 0 -250 ppm NaOCl at various time points (orange bars) and on stainless steel coupons after 30 minutes in the respective washes. Transfer of* Lm *from the surfaces of stainless steel coupons to spinach is indicated by a "+" and no transfer by a "–" sign.

All the NaOCl-containing wash solutions prevented the transfer of *Lm* except for the 0-ppm wash solution. Also, none of the wash solutions except the 0-ppm had *Lm* counts above the detection limit (2.6 log_{10} CFU. Residual *Lm* was detected on

the coupons for all the NaOCl concentrations used except for treatments with 250 ppm after 20 min. This outcome shows that it is nearly impossible to completely inactivate *Lm* cells in a biofilm at concentrations often used by produce processors.

Chapter 5

CONCLUSION AND FUTURE WORK

In general, concentrations of antimicrobials needed to completely inactivate *Lm* attached to surfaces was more than 10-fold higher than those required for inactivation of *Lm* in a planktonic state. Also, information obtained from this study indicates that *Lm* biofilms cannot reliably be inactivated by antimicrobial solutions at concentrations usually found in produce rinse water. Even though it is possible for antimicrobial solutions commonly found in produce water to prevent transfer of *Lm* released from biofilm to surfaces not coated with bacteria or organic matter, usually higher concentrations would have to be applied to achieve the same effect for surfaces or processing systems coated with organic matter or microorganisms.

The data from this study also puts emphasis on the need to prevent biofilms from forming because they serve as harbors for *Lm*. However, in situations where biofilms have already been established, it is likely that prevention of transfer to other surfaces and produce could be possible as long as the recommended concentrations of antimicrobials are closely monitored and maintained in the produce rinse water.

Previous studies have shown that *Lm* is present to various degrees in soils and can remain viable there for extended periods of time (Dowe et al., 1997; Fenlon,

1999; McLaughlin et al., 2011). Produce harvested in the field and perhaps even packaged on site as well as transport equipment can carry soil into processing facilities. Given the complex structure of particles found in soil and the presence of organic matter, it is likely that *Lm* and other pathogens enjoy a level of protection from antimicrobials that they do not when associated with smooth surfaces such as stainless steel of plastics. Therefore, future studies need to be done with soils to determine the concentrations of antimicrobials that are effective against *Lm* associated with soil matter.

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