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FATE OF *LISTERIA MONOCYTOGENES* ON INTACT AND WOUNDED APPLES
DURING STORAGE AND IN A SIMULATED DUMP TANK

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by

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ABSTRACT

The effect of fruit carnauba wax and shellac fruit coatings on the fate of *Listeria monocytogenes* inoculated onto whole fresh apples during storage was studied. Little information exists on the effectiveness of commercial fruit coatings for reducing *L. monocytogenes* populations on fruit. There is also little information on the effect of post-storage parameters, such as temperature, relative humidity, apple variety, and wound damage on fate of *L. monocytogenes* on stored apples nor the degree to which cross contamination is limited when apples are submerged in sanitizer solutions, such as occurs in apple dump tanks and flumes. To address these issues, studies were done to determine: 1) if commercially-available carnauba wax or shellac coatings affect *L. monocytogenes* growth; 2) the fate of *L. monocytogenes* populations on intact and wounded fresh apples during refrigerated or ambient temperature; and 3) the level of *L. monocytogenes* destruction and cross-contamination that occurs in a simulated dump tank.

In the first study, a plate overlay assay method was used to determine if carnauba wax or shellac formulations had any effect on growth and survival of *L. monocytogenes*. Solid pellets were prepared by thoroughly drying the coatings in plastic molds to eliminate any potential antimicrobial effects of the carrier solvents present in the formulations. Results from this study showed no zones of inhibition or signs of growth enhancement after 24 and 48 hours incubation, thus indicating that neither coating had negative or positive effects on *L. monocytogenes* growth.
To follow up on these preliminary results, the fate of *L. monocytogenes* during refrigerated (4°C) storage of coated or un-coated apples for up to 28 days was determined. In addition to coating effects, variables tested were incorporation of 0.1% lauric arginate into the coating formulations, direct application of 0.1% aqueous lauric arginate onto apples prior to storage, and skin wounding. Wounds were created to simulate damage to apples that could occur during transportation or movement along the packing line. Significant (p<0.05) decreases in *L. monocytogenes* were observed for all treatments and the control during the 28-day storage period at 4°C and 50% RH. There were no significant differences between the two coating types and the control, nor were there differences between the two coating types, thus confirming the results of the plate assay. Incorporation of 0.1% lauric arginate in either coating formulation only slightly increased the rate of *L. monocytogenes* destruction on intact inoculation sites, but not on the wounded sites. Overall, the aqueous lauric arginate resulted in a greater reduction rates compared to the same antimicrobial emulsified into the fruit coatings.

In another experiment of this study, the effects of storage temperature (4 or 20°C), storage relative humidity (50 or 89 % RH), and apple variety (Fuji or McIntosh) on intact or wounded inoculation sties were studied. No significant differences in *L. monocytogenes* populations were observed after 28 days for either storage temperature or RH value. No varietal differences were observed, despite differences in tissue pH, titratable acidity, and total solids. Based on the results from the two coating experiments, it was concluded that *L. monocytogenes* population reductions that occurred on apples stored at 4°C storage and 50% relative humidity, but not on apples were stored at 4°C and 89% relative humidity, could be attributed to known negative
effects that relative humidity reductions have on *L. monocytogenes* survival. Interestingly, at ambient temperature (20°C) storage and 50% relative humidity, the humidity effect did not occur.

In the third study, three inoculated apples were placed into a simulated dump tank containing water or various sanitizer solutions along with three other non-inoculated apples and immersed for 5 minutes. *L. monocytogenes* populations were determined for both the inoculated apples and the three non-inoculated apples at the end of the immersion period. The objective of this study was to determine the extent to which selected sanitizers, approved for commercial use, could reduce populations on inoculated apples and also to what extent these sanitizers could prevent cross-contamination of *L. monocytogenes* from the inoculated apple to the non-inoculated apples. The sanitizers and concentrations studied were sodium hypochlorite (100, 150, or 200 ppm OCl⁻ adjusted or not adjusted with citric acid to pH 6.8), acidified sodium chlorate (chlorine dioxide at 5 ppm), and a series of peroxyacetic acid (PAA) / hydrogen peroxide formulations (60, 80, or 100 ppm PPA).

Sodium hypochlorite did not significantly (p<0.05) reduce of *L. monocytogenes* populations on inoculated apples at the un-adjusted pH of 8.7-9.0. However, in solutions adjusted to pH 6.8, Oxidation Reduction Potential (ORP) values doubled and microbial destruction of the pathogen was proportionally higher as compared to the control. Addition of unadjusted and adjusted pH sodium hypochlorite decreased *L. monocytogenes* cross contamination to non-inoculated apples, except for 100 ppm at pH 8.7. Similarly, to sodium hypochlorite solutions adjusted to pH 6.8, peroxyacetic acid/hydrogen peroxide sanitizers (SaniDate products) were significantly more effective
at limiting cross contamination as compared to the control, with the lone exception being SaniDate FD at 60 ppm PAA. In addition, SaniDate products were more effective at reducing *L. monocytogenes* populations than high pH sodium hypochlorite solutions. However, complete prevention of cross contamination was not possible and concentration was not a significant effect. Despite only partial control of *L. monocytogenes*, addition of sanitizers to dump tank or flume water is a recommended industry practice.

Overall, the results from the studies showed that complex organic coating compounds in apple coating do not serve as a nutrient source for *L. monocytogenes*. This is a good thing for the industry which seeks to keep *L. monocytogenes* populations as low as possible. However, the little or no population reductions observed suggest that the industry cannot rely on long term storage to reduce high levels of *L. monocytogenes* contamination. Sanitizers were significantly more effective at limiting cross contamination than plain wash water. However, there was incomplete pathogen reduction and always some level of cross contamination in the simulated dump tank. While sanitizers can be relied on to deal with low pathogen levels, apple growers must engage in Good Agricultural Practices that prevent contamination, thus minimizing *L. monocytogenes* risks. Since only a 5-minute immersion time was studied, future studies might consider the effects of higher sanitizer immersion times that might increase *L. monocytogenes* reductions on fresh apples.
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INTRODUCTION TO THE THESIS

Due to a large portion of the population's fascination with health and nutrition, fresh produce, including apples, are widely consumed throughout the world. Over 4 million tons of apples are produced in the U.S. per year, with an annual per capita consumption rate of 15-16 pounds. Apples are very high in phenolics, thus making up a significant component of phenolic compounds in the American diet (Wolfe et al. 2003). Apples have been known to have strong antioxidant activity, inhibit cancer cell proliferation, decrease lipid oxidation, and lower LDL cholesterol (Wolfe et al. 2003). Reduced risks of cancers, cardiovascular disease, asthma, and Type 2 diabetes have been reported from regular consumption of apples (Wolfe et al 2003). However, there have been several L. monocytogenes recalls and outbreaks linked to contaminated apples including the Del Monte Gala sliced apple recall of 2014 in Pennsylvania (FDA 2015a) and the Bidart Brothers caramel apple outbreak of 2015 in Bakersfield, California (CDC 2015b).

Common methods used to limit contamination of apples include adding sanitizers to dump tanks, flumes, and spray washes. Laboratory studies have shown population reductions of 5 logs or greater on some produce treated with sanitizers (Rodgers et al. 2004). After apples are washed, they are typically fan dried and then coating with commercial wax or shellac formulations. Antimicrobials have been incorporated into coatings to control plant pathogens, although this strategy has not been used to reduce
*L. monocytogenes* populations on fresh apples. Thus, this is an area that needs to be studied.

Temperature and relative humidity are known to have an effect on microbial populations, including *L. monocytogenes*. The effects of relative humidity on *L. monocytogenes* populations on apples over an extended storage period have not been studied. However, previous studies have shown that *L. monocytogenes* population reductions on parsley (Dreux et al 2007) or polypropylene (Zoz et. 2016) surfaces are greater at lower relative humidity values. Fresh apples are typically stored between 4 and 9°C at between 50 and 95% relative humidity (Simcox et al 2001). Recently, Sheng et al (2017) demonstrated that *L. monocytogenes* on apples stored at 4°C were only slightly changed over time with no significant differences reported after 4 weeks of storage. Studies on sliced apples have shown that *L. monocytogenes* is able to survive over time and that populations can slightly increase during storage at 10 and 20°C (Conway et al 2000). No existing studies have reported the combined effects of low and high relative humidity and low and high temperature. Thus, this is a research area that needs to be studied.
CHAPTER 1

STATEMENT OF THE PROBLEM

The fresh produce industry is concerned about the potential for contamination of fruits and vegetables with human pathogens, including *Listeria monocytogenes*. There have been several recent recalls and outbreaks linked to contaminated apples and apple products. Current attempts to eliminate the incidence of such recalls and outbreaks are primarily centered around adding commercial sanitizers to dump tanks and flumes before they are washed with overhead sprays, fan-dried, and coated with wax or shellac. It is also of concern that apples are stored at varying humidity levels that might allow for the sustenance of *L. monocytogenes*, if present.

Therefore, the research objectives of this project are as follows:

**Objective 1:** Determine whether carnauba wax and/or shellac coatings have any effects on growth and survival of *Listeria monocytogenes*.

**Objective 2:** Determine any effects that fruit coatings, coating antimicrobial emulsions, aqueous antimicrobial solutions, wounds, temperature and relative humidity have on the growth and survival of *L. monocytogenes* on whole apples during long term storage.

**Objective 3:** Determine what effect commercial sanitizers have on *L. monocytogenes* populations on fresh apples in a simulated dump tank and the extent to which cross contamination of *L. monocytogenes* can occur.
CHAPTER 2:

LITERATURE REVIEW

2.1 Trends in Fresh Produce Consumption

Increasing concerns among consumers for improving and maintaining good health have resulted in greater consumption of fresh fruits and vegetables (Slavin and Lloyd 2012). Total worldwide production of apples is over 80 million tons each year with an approximate value of $14 billion (USDA Foreign Agricultural Service 2015). In the United States, 7,500 apple producers grow 240 million bushels of apples on average each year (U.S. Apple Association 2018). High levels of health benefitting phenolics in apples account for their significant contribution of these compounds in the American diet (Wolfe et al. 2003). Apple consumption has shown to inhibit HepG2 human liver cancer cells, as well reduce the risk of asthma, cardiovascular disease, and diabetes (Boyer and Liu 2004). Apple peels have particularly high antioxidant and antiproliferative activity, decrease lipid oxidation, and lower LDL cholesterol (Wolfe et al. 2003).

2.2 Listeria Recalls and Outbreaks Involving Produce

2.2.1 Fresh Produce

Cases of listeriosis have long been associated with unpasteurized dairy products and contaminated ready to eat meat products (Pamer 2004). With the increased consumption of fresh produce in recent years, it is not unexpected that there have been
several *L. monocytogenes* recalls and outbreaks attributed to fresh fruits and vegetables.

In 1979, a *L. monocytogenes* outbreak involving raw celery, tomatoes, and lettuce consumed in Boston, Massachusetts caused 20 illnesses (all requiring hospitalization) and 5 deaths (Ho et al. 1986). Epidemiological analysis revealed that serotype 4b epidemic clone 1a was the cause. Although contaminated produce was epidemiologically implicated, it was not microbiologically confirmed (Ho et al. 1986). Unlike more recent outbreaks, there was no available information capable of tracing the exact source of contamination.

In 2010, a listeriosis outbreak attributed to diced celery processed in Colorado spanned 7 months with a total of 10 patients infected, resulting in 5 deaths (Gaul et al. 2013). The outbreak was traced back to contaminated machine-cut diced celery used to prepare a chicken salad served at the hospitals. Contamination of the production facility environment, including the slicing equipment, was found. The identified *L. monocytogenes* outbreak strain was found in 7 of the 10 bags of the finished product that was collected (Gaul et al. 2013). Environmental swabs collected from floors, surfaces, and equipment in every room of the facility tested positive for *L. monocytogenes* (CDC 2017).

In 2011, a large multistate listeriosis outbreak attributed to contaminated cantaloupe from Jensen Farms in Granada, Colorado resulted in 47 invasive illnesses, 30 deaths, and one miscarriage and was somewhat uncommon in that there were multiple *L. monocytogenes* serotypes involved, including 1/2a and 1/2b (CDC 2012). Also
uncommon was the fact that there were five widely differing pulse field gel electrophoresis combinations among the isolates (Laksanalamai et al. 2012). The outbreak was linked to *L. monocytogenes* contamination of processing equipment used at the production facility, as well as the lack chlorination of the water used to wash the cantaloupes (CDC 2012).

In 2014, mung bean sprouts grown in Illinois and Michigan caused five cases of listeriosis requiring hospitalization and two deaths occurred (CDC 2015a). A thorough FDA inspection and testing of the facility where the sprouts were grown yielded *L. monocytogenes* positive isolates from the mung bean sprouts, the irrigation water used from sprout production, as well as environmental swabs (CDC 2015a). Pulsed field gel electrophoresis and whole genome sequencing of the positive isolates showed that they were closely related to the isolates obtained from the infected patients (Garner and Kathariou 2016). Further inspection of the implicated processing facility revealed multiple instances of unsanitary conditions and subpar maintenance of production equipment. Subsequent inspections conducted several months later again yielded the same outbreak strain from environmental samples, and the facility was eventually shut down due to persistent *L. monocytogenes* contamination (CDC 2015a).

In 2016, the U.S. Food and Drug Administration (FDA), the U.S. Centers for Disease Control and Prevention (CDC), and state and local officials investigated a multi-state outbreak of listeriosis (FDA 2016, CDC 2016). According to CDC, 19 people were infected with *Listeria monocytogenes* in nine states and one person from Michigan died. According to CDC, whole genome sequencing (WGS) was performed on clinical isolates from all ill people and showed that the isolates were highly related genetically.
2.2.1 Apples and Apple Products

Several recalls and outbreaks have been linked to contaminated tree fruit. In July 2014, California-grown whole peaches, nectarines, plums, and pluots were recalled after *L. monocytogenes* was detected on fruit shipped to Australia (Jackson et al. 2015). Also in 2014, Del Monte fresh cut fruit mixes containing cut Gala red apples tested positive for *L. monocytogenes* were traced to a single packing house in Pennsylvania (FDA 2015). In both of these instances, the ultimate source of the *L. monocytogenes* remains unknown. Although contamination within the packing house is suspected, it is uncertain whether the pathogen contamination occurred in the orchard or the packing facility.

In 2015, *Listeria monocytogenes*-contaminated caramel apples packed by Bidart Brothers in Bakersfield, California caused 35 illnesses in 12 states, with 34 hospitalizations and 7 deaths occurring (FDA 2015b, CDC 2015b). Whole genome sequence analysis showed that the packing house isolates closely matched clinical strains found in those who had become ill (CDC 2015b).

Most recently in 2017, whole apples packed in Michigan were recalled after routine testing revealed the presence of *Listeria monocytogenes* (FDA 2017a). Immediately after, apples slices prepared from the Michigan apples were recalled as a precautionary measure (FDA 2017b). No illnesses were reported for either of these events. These recalls and outbreaks have led to a greater awareness of the risk of *L. monocytogenes* contamination of tree fruit, including apples and apple slices.
2.3 *Listeria monocytogenes*

**2.3.1 General Characteristics of *L. monocytogenes***

*L. monocytogenes* is Gram-positive, non-sporeforming, facultatively anaerobic organism (Farber and Peterkin 1991). It is catalase positive, oxidase negative, and causes an illness known as listeriosis. Human exposure to *Listeria monocytogenes* comes from consuming contaminated food, such as unpasteurized dairy products, ready to eat meat products (Pamer 2004), as well as contaminated fresh produce. Human exposure to *Listeria monocytogenes* is very common. However human development of invasive listeriosis is not very common, as *L. monocytogenes* invades through the intestines first and later multiplies intracellularly (Schuchat et al. 1991). Listeriosis infections are most common in infants, elderly, and immunocompromised individuals, or those with significantly weaker than normal defenses against bacterial infection (Acheson 2000). These people include cancer patients, HIV/AIDS patients, as well as those suffering from other diseases. Although both males and females are capable of acquiring listeriosis, infections are predominant in males (Acheson 2000). *Listeria monocytogenes* is generally susceptible to most drugs used to treat human bacterial infections, although clinical *L. monocytogenes* strains have shown resistance to the antibiotics chloramphenicol, erythromycin, streptomycin, tetracycline, vancomycin and trimethoprim, thus indicating a public health risk (Reis et al. 2011). *L. monocytogenes* accounts for less than 1% of all foodborne illnesses, but accounts for around 28% of deaths from foodborne illness, thus signifying the seriousness of *L. monocytogenes* human infection.
2.3.2 Evolutionary Lineage

There are 12 serotypes of *L. monocytogenes* known to exist; 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4b, 4c, 4d, 4e, and 7. *Listeria* virulence is serotype-dependent, as some serotypes have been associated with more serious symptoms than others (Morobe et al. 2012). Serotypes 1/2a, 1/2c, 1/2b and 4b are involved in 98% of reported human cases. Serotype 4b is especially prevalent in *L. monocytogenes* outbreaks, as it is involved in 64% of human cases. Three evolutionary lineages exist of *L. monocytogenes* exist. Lineage I includes the serotypes 1/2b, 3b, 4b, and 4d. Lineage II includes the serotypes 1/2a, 1/2c, 3a, and 3c. Lastly, lineage III includes the serotypes 4a, 4c as well as 4b (Morobe et al. 2012).

2.3.3 *L. monocytogenes* Survival and Persistence

Unlike most other foodborne pathogens, *L. monocytogenes* is capable of persisting in a wide range of environmental conditions. One major adaptation is the ability to survive and grow at refrigeration temperatures (4°C), a trait that is explained by its ability to modify the composition and therefore the fluidity of fatty acids in its cytoplasmic membrane (Sinensky 1974). The cytoplasmic membrane of *L. monocytogenes* is primarily composed of branched chain fatty acids (Mastroncolis et al. 2005), such as anteisoC15:0, anteisoC17:0, and isoC15:0. Its ability to increase the ratio of anteisoC15:0 fatty acids under refrigeration conditions accounts for its ability to grow at refrigeration temperatures (Wilkinson et al. 2015). This process which maintains membrane fluidity at low temperatures is known as homeoviscous adaptation (Sinensky 1974).
Juneja and Davidson (1993) showed that *L. monocytogenes* grown in the presence of exogenously added C14:0 or C18:0 had higher resistance to the antimicrobials sodium chloride, tertiary butylhydroquinone, methyl paraben, and propyl paraben, whereas *L. monocytogenes* grown in the presence of exogenously added C18:1 led to increased sensitivity to those same antimicrobials (Juneja and Davidson 1993). This observation illustrates that susceptibility of *L. monocytogenes* to antimicrobial agents is directly related to cell membrane fatty acid composition.

### 2.3.4 *L. monocytogenes* and Biofilms

One of the major reasons why *L. monocytogenes* is difficult to eradicate from food processing facilities is its ability to form biofilms. A biofilm is an assemblage of microbial cells that is usually irreversibly attached to a surface, (i.e. not easily removed by gentle rinsing) and that is enclosed in a polysaccharide matrix (Donlan 2002). Bacteria, including *L. monocytogenes*, naturally exist by attaching to both living and non-living surfaces (Carpentier and Surf 2011). The process of surface attachment is irreversible once extracellular polymeric substances (EPS) bind to surfaces. EPS plays a major role in the persistence of biofilms, in that they provide a structure for the biofilm (Donlan 2001).

Biofilms are of serious concern to the food industry, largely due to their ability to resist the effectiveness of antimicrobial agents (Buchanan et al. 2017). In order for antimicrobial compounds to reach pathogens encased in a biofilm, they must be able to diffuse through the EPS before they can reach the microorganism (Donlan 2001). EPS lowers the rate of diffusion by either reacting with the antimicrobial molecules or by
lowering the rate of transport of such molecules (Pan et al. 2006). Thus, the surrounding environment within a biofilm not only protects the microorganism, but further supports its growth. This concept is especially important to food processors and reinforces the need to clean and sanitize food production areas appropriately (Pan et al. 2006).

2.4 The Apple Packing Process

Harvested apples are placed into bulk bins in the orchard before they are transported to a packing or storage facility. Apples are loaded onto the packing line by immersing the bins into a dump tank or by directly feeding them onto a receiving conveyer belt (Simcox et al. 2001). They are then moved through a series of rotating brushes under a rinse water spray. Then then are then conveyed under a series of fan driers and rotary sponges, after which they are sprayed with food-grade fruit coatings, which increase shelf life and improve visual appearance. Apples may then sorted by selecting out oversized, undersized, and damaged fruit, which are often later used for livestock feed, canning, applesauce, cider, and jams (Simcox et al. 2001, Mitcham and Mitchell 2002). They are also visually inspected for color, bruising, and rot. After sorting, apples are then packaged into a variety of containers, primarily boxes, bags, and bins, with the more delicate apples being placed into pressed paper fiber trays that are layered into the boxes. Apples are boxed by size, ranging anywhere from 38 to 150 apples per box, with the most common size being 88 apples per box (Simcox et al. 2001, Mitcham and Mitchell 2002).
2.5 Coatings and Films

An edible coating is a thin layer of coating applied to the surface of a particular food (McHugh 2000), whereas an edible film is a thin preformed layer of edible material that can be placed on or between food components (McHugh 2000). Fruit coatings act as protective films on produce by raising tissue carbon dioxide levels, lowering oxygen levels and lowering metabolic activities. Such attributes can lead to greater resistance to plant pathogens, thus increasing the produce shelf life (Goncalves et al. 2010). Edible films and coatings can protect foods from contamination from microorganisms and prevent quality loss due to mass transfer from moisture. Coatings also can be used as a vehicle for incorporating antimicrobials, antioxidants, vitamins, minerals, enzymes, and probiotics (Skurtys et al. 2011). Coatings consist of three categories; hydrocolloids, lipids (waxes are in this category), and composites. The most widely used fruit coatings are carnauba wax and shellac (Goncalves et al. 2010).

2.5.1 Carnauba Wax

Carnauba wax is naturally-obtained from the wax of the leaves of the palm Copernicia prunifera. It has been used in coating the surface of fruits and vegetables since the 1950s (Bai et al. 2003). Carnauba wax has been recognized as non-toxic and safe to use on fresh produce, though no chronic toxicity or carcinogenicity studies are available (European Food Safety Authority 2012). Carnauba wax is relatively hydrophilic, consisting of alipathic wax esters, alpha hydroxyl esters, and alipathic diesters. Wax esters make up 84% of carnauba wax, while hydrocarbons make up only 2% of carnauba wax (EFSA 2012). The maximum permitted use on apples is only 200
mg/kg, less than that allowed on confectionary products (500 mg/kg) and on chewing gum (1200 mg/kg). In a study conducted by Jo et al., (2014) population reductions of both *L. monocytogenes* and *E. coli* O157:H7 were observed when they were inoculated into tubes containing a liquid carnauba wax formulation. *L. monocytogenes* reductions of 1 log were observed after 24 hours storage. It should be noted however, that when Jo et al (2014) performed this study, it was not known if the carnauba wax itself caused the population reductions that took place, or if the reductions that took place were due to the solvent present in the wax. The exact composition of the solvents in the wax itself are unknown.

Jo et al. (2014) also evaluated the effect of essential oils (tea tree oil, lemongrass oil, and tea tree oil) emulsified in carnauba wax on *L. monocytogenes* and *E. coli* populations. Lemongrass oil added to carnauba wax provided the greatest reductions of the two microorganisms which were significantly greater than carnauba wax alone, thus indicating a possible future antimicrobial application. Apples were not tested in this study (Jo et al. 2014), so one would not know what interactions apples would have with the wax-antimicrobial emulsion and whether or not that the wax-antimicrobial emulsion would significantly reduce *L. monocytogenes* on apples if applied. Because such positive results were obtained from that study, the study serves as an excellent springboard from which to carryover that same emulsion formed and apply it to *Listeria monocytogenes*-inoculated apples to observe if one will get the same beneficial effects to occur when antimicrobials are incorporated into apple coatings. It should be noted, however, that the authors of the paper did not remove the solvent from the carnauba
wax for this experiment, which could possibly explain at least some of the reduction obtained.

A study by Kenney et al. (2002) looked at the effects of carnauba wax fruit coatings on *E. coli* O157:H7 and *Salmonella Muenchen* (i.e. *Salmonella enterica* subsp. *enterica* serovar *Muenchen*) populations on fresh apples during storage for up to 12 weeks. Relative humidity conditions during storage at 2 and 21°C were 43 and 74%, respectively. Population reductions for *S. Enterica* on apples stored at 2°C for two different commercial wax formulations were 4.62 and 4.61 log₁₀ CFU/apple after 12 weeks. Reductions were greater compared to untreated or water rinsed apples which were 0.39 and 1.86, respectively, after 12 weeks. For apples stored at 21°C, no significant differences in *Salmonella* populations occurred after 6 weeks. For *E. coli* O157:H7, populations reductions for the two coatings were 4.62 and 3.35 log₁₀ CFU/apple after 6 weeks at 2°C which were greater than 6 week untreated or water rinsed apple reductions of 0.63 and 2.37 logs, respectively. In contrast to the behavior of *S. Enterica* at 21°C, populations for the two coatings were reduced by 1.77 and 1.44 log₁₀ CFU/apple after 6 weeks at 21°C and untreated or water rinsed apple reductions of 5.58 and 0.05, respectively, were achieved. No explanation for the varying results for each pathogen and the temperatures studied was given and possible effects due to humidity differences were not discussed. It will be interesting to compare these results with storage effects on *L. monocytogenes* populations given that *L. monocytogenes* is a Gram-positive microorganism and *E. coli* and *S. Enterica* are Gram-negative organisms.
2.5.2 Shellac

Shellac coating is obtained from a natural resin deposited onto trees by the female lac bug, *Kerria lacca*. The raw shellac is scraped from the trees and filled into canvas tubes for subsequent heating. After being heated, the shellac liquefies and begins to come out of the tubes as a thick, sticky substance. The substance is then dried, crushed into a fine powder, and mixed with ethanol to produce liquid shellac. Although the exact chemical composition of shellac is unknown, it consists in general of hydroxyl fatty esters and sesquiterpene acid esters, both with a molecular weight of approximately 1000 grams per mole. The major constituents of shellac are aleuretic acid, r-butolic acid, shellolic acid, and jalaric acid (Yates and Field 1970). The chemical composition of shellac is a function of the source and time of harvest of the sticklac used, which can bring about variability in shellac that is produced. The physical properties of shellac can also vary, as the reported melting point can range anywhere from 77° to 120°C. Shellac is soluble in numerous hydrophilic solvents, including ethanol, methanol, glycols, glycol ethers, and alkaline water (Yates and Field 1970). Like carnauba wax, shellac is used as a coating on fruit to prolong storage and shelf life (Bai et al. 2003). Shellac similarly lowers fruit metabolic activity by raising tissue carbon dioxide levels and lowering oxygen levels (Goncalves et al. 2010). When specifically applied to Red Delicious apples, shellac imparts high gloss, effectively hide bruises, and creates a modified atmosphere that preserves firmness and leads to a prolonged shelf-life (Bai et al. 2002).
2.6 Sanitizers and Antimicrobials Used

2.6.1 Peroxyacetic Acid

Peroxyacetic acid (PAA) is an organic peroxide sanitizer used in meat and poultry processing plants, cheese and dairy processing plants, and on fresh produce. Industrial production of PAA is through autoxidation of acetaldehyde. It also is formed from the treatment of acetic acid with hydrogen peroxide, though PAA is a much weaker acid than the parent acid, acetic acid (PAA has a pKa of 8.2; acetic acid has a pKa of 4.3) (Buchanan 1985). PAA is extremely unstable and readily decomposes to its original constituents of acetic acid and hydrogen peroxide when dissolved in water for use as an aqueous solution (USDA 2016). These disintegration products will further break down into water, oxygen, and carbon dioxide, which are all non-toxic, thus further indicating that it is a non-toxic sanitizer safe for use on produce. PAA is a strong oxidizing agent with a redox potential of 1.762 E_v. (Buchanan 1985). The highly effective antimicrobial action of this sanitizer can be explained by the transfer of electrons to the outer cell membrane of bacteria where oxidation severely disrupts cell membrane transport from taking place (Buchanan 1985). The Environmental Protection Agency (EPA) has established a limit of 100 ppm of PPA for use on fresh produce (Title 40 CFR Section 180.1196). Previous work done by Rodgers et al. (2004) showed that PAA used at a concentration of 80 ppm for 5 minutes produced population reductions of 5-logs or greater of dip-inoculated *Escherichia coli* O157:H7 and *L. monocytogenes*. Contradicting results were observed by Wisniewsky et
al., who only observed a 3 log reduction from 80 ppm PAA and required a PAA concentration of 1280 PPM to clear the 5 log reduction threshold (Wisniewsky et al. 2000).

2.6.2 Hydrogen Peroxide

Hydrogen peroxide is the simplest existing peroxide with a very strong oxidizing agent with a slightly higher viscosity than water. It is most commonly used in aqueous solutions (Finnegan et al. 2010). Like several other sanitizers used in aqueous solution, hydrogen peroxide is quite unstable and deteriorates over time to form water and oxygen. Decomposition is accelerated by substantial increases in temperature. The antimicrobial activity of hydrogen peroxide is dependent on the release of nascent oxygen, which has a very powerful oxidizing effect on microorganisms, thus altering the chemical composition and molecular structure of the bacterial cell leading to overall loss of cellular strength (Maris, 1995). Hydrogen peroxide removes electrons from chemical groups that are susceptible to oxidation which in turn is reduced in the process (Finnegan et al. 2010). Hydrogen peroxide also has the ability to destabilize spores (Maris 1995), but does not completely destroy them, suggesting that it is not an effective antimicrobial agent against microorganisms that are able to form spores (Finnegan et al. 2010). Previous work done by Venkitanarayanan et al. (2002) involved spot-inoculation of apples, oranges, and tomatoes with five-strain mixtures of *E. coli* O157:H7, *Salmonella enteritidis*, and *L. monocytogenes* near the stem end and subsequent submersion into sterile deionized water containing 1.5% lactic acid plus 1.5% hydrogen peroxide for 15 minutes at 40°C. The treatment reduced levels of the pathogens by 5 log CFU per fruit, whereas washing in deionized water decreased the pathogens by only
1.5 to 2.0 log CFU per fruit. They observed that substantial populations of the pathogens survived in the control wash water, whereas no *E. coli* O157:H7, *Salmonella enteritidis*, or *L. monocytogenes* cells were detected in the chemical treatment solution (Venkitanarayanan et al. 2002).

### 2.6.3 Peroxyacetic Acid/Hydrogen Peroxide Formulations

The primary reason for combining peroxyacetic acid and hydrogen peroxide is that peroxyacetic acid is very unstable and readily breaks down. Addition of hydrogen peroxide slows the deterioration process and drives the reaction in the reverse direction (Vandekinderen et al., 2009). Multiple sanitizers consisting of this formulation of peroxyacetic acid and hydrogen peroxide are manufactured by several companies including BioSafe Inc., which includes the sanitizers SaniDate 5.0, SaniDate 12.0, SaniDate 15.0, and SaniDate FD. Peroxyacetic acid concentrations of these sanitizers range from 5.3% to 15% and hydrogen peroxide concentrations range from 5% to 23%.

### 2.6.4 Sodium Hypochlorite

Sodium hypochlorite is another commonly used sanitizer used not only in the food industry, but also in healthcare facilities, as well as other settings. According the U.S. Code of Federal Regulations, no-rinse application of sodium hypochlorite on food contact surfaces is allowed, provided that the aqueous solution does not contain more than 200 ppm chlorine (Beuchat et al. 1998), although a maximum of 2000 ppm sodium hypochlorite is allowed in wash water (Beuchat et al. 1998). A 200 ppm sodium hypochlorite solution is equivalent to a tablespoon of typical commercial bleach containing 5.25% sodium hypochlorite per gallon of water. It is important to limit the
concentration of sodium hypochlorite used, due to the fact that sodium hypochlorite is a very strong oxidizer; solution concentrations greater than 500 ppm chlorine can be corrosive to some metals and alloys.

Sodium hypochlorite has a multifaceted mechanism of action that involves the release of hydroxyl ions due to its high pH of 12.5 that alters the integrity of the cytoplasmic membrane by either the chemical destruction of organic components or the degradation of cytoplasmic membrane fatty acids or phospholipids (Estrela et al. 2002). Sodium hypochlorite also interferes with cellular metabolism through the formation of chloramines through an amino acid chloramination reaction. Being a strong oxidizer, sodium hypochlorite promotes an irreversible bacterial enzymatic inhibition through the reaction of chlorine with amino groups and also through the irreversible oxidation of sulfhydryl groups of bacterial enzymes, such as cystein (Estrela et al. 2002). The antimicrobial action of sodium hypochlorite involves the promotion of irreversible inactivation of bacterial enzymatic sites originating from the release of hydroxyl ions and also through chloramination action (Estrela et al. 2002). Work done by Rodgers et al. (2004) showed that the benefits of chlorine based sanitizers on L. monocytogenes populations on apples. Chlorine trisodium phosphate (both 100 and 200 ppm) and chlorine dioxide (both 3 and 5 ppm) used for 5 minutes produced a 5-log reduction or greater of Escherichia coli O157:H7 and Listeria monocytogenes on previously been dip-inoculated apples and other produce (Rodgers et al. 2004).
2.6.5 Chlorine Dioxide

A less-commonly used chlorine-based sanitizer that is sometimes used on produce is chlorine dioxide. Like sodium hypochlorite, chlorine dioxide is a very strong oxidizing agent, which gives it its bactericidal and antiseptic properties. Chlorine dioxide readily breaks down into chlorite when used as a disinfecting agent, which is toxic to red blood cells at concentrations of 10 mg/kg bodyweight and higher (USDA 2006). However, no toxic products occur when chlorite mixes with organic materials, making it safe to use on fresh produce (USDA 2006). A unique property of chlorine dioxide is that it is an explosive gas in its natural state (Hartshorn 1978). Because of this, it is generated from more stable constituents (such as sodium chlorite and citric acid) prior to use. Rodgers et al. showed that aqueous chlorine dioxide concentrations of 3 and 5 ppm applied for 5 minutes are necessary to achieve a 5-log reduction of *L. monocytogenes* populations on whole apples (Rodgers et al. 2004). Du and Linton 2002 demonstrated over a 5-log reduction of *L. monocytogenes* at both 4 mg/L and 8 mg/L chlorine dioxide on apples. However, Wisniewsky et al. (2000) observed only 3 log *L. monocytogenes* reductions for chlorine dioxide concentrations of 5 ppm. It should be noted, however, that the EPA has a limit of 3 ppm for chlorine dioxide used in produce wash water (Title 21 CFR Section 173.300).

2.6.6 Lauric Arginate

Lauric arginate has been used as an antimicrobial on RTE poultry products such as hams and frankfurters, but more recent use is with fresh produce (Taormina, Dorsa 2009, Stopforth et al. 2009). It is a cationic surfactant, composed of lauric acid, L-
arginine, and ethanol. This composition is significant because the cationic and hydrophilic nature of lauric arginate may in some cases reduce its antimicrobial effectiveness due to its ability to bind to anionic and hydrophobic components present in some food systems (Bonnaud et al. 2010). Lauric arginate applications may also be limited due to the fact that it precipitates from solution at pH values greater than 4.5 and at high ionic strengths (Asker et al. 2009). Nevertheless, varying levels of antimicrobial activity over a pH range of 3 to 7. Lauric arginate has a low oil-water equilibrium partition coefficient and a high partition coefficient of greater than 10, meaning that it concentrates in the water phase of products, which is where most bacterial action takes place (Saini et al. 2013). Lauric arginate is a GRAS (Generally Recognized as Safe) compound and is of low toxicity to humans. An upper limit of 225 mg/kg was established by the USFDA in 2005 (World Health Organization 2009). It is rapidly metabolized in humans and broken down into its components lauric acid and arginine (Hawkins et al. 2009). Lauric arginate acts directly on the cytoplasmic membrane of microorganisms. It disrupts the cytoplasmic membrane, thus causing an unstable membrane bilayer and altering cellular metabolic processes and ultimately altering the cell cycle, though it does not cause cell lysis (Stopforth et al. 2009). The effectiveness of lauric arginate against *L. monocytogenes* has previously been studied Pattanayaiying et. al (2014) and it may be useful when incorporated in to packaging films (Pattanayaiying et al. 2015). Antimicrobials have been used in aqueous solutions, but not incorporated into coatings, such as carnauba wax and shellac. In this thesis, the effectiveness of the incorporation of lauric arginate into carnauba wax and shellac against *L. monocytogenes* populations on whole fresh apples will be evaluated.
2.6.7 Sanitizer Accessibility

It is widely known that chlorine-based, peroxyacetic acid-based, and hydrogen peroxide-based sanitizers are highly effective at reducing pathogens on the surfaces of fresh produce, when used at their respective CFR (Code of Regulation) recommended amounts. Though pathogen reductions of 5 logs and above have been reported from the application of such sanitizers when applied to produce surfaces, effectiveness steeply declines when applied to crevices, creases, pockets, and skin openings on produce. A study done by Pao et al. (1999) reported a 2 log reduction of *E. coli* on an orange surface, but only a 1 log reduction on a stem scar region from sodium triphosphate. Du and Linton (2002) also reported similar findings on apples, with a 5.5 log reduction of *L. monocytogenes* on apple surfaces from chlorine dioxide, but only a 3.2 log reduction on stem regions and 3.6 log reduction on calyx cavities. It should be noted, that oranges have much shallower stem regions than apples, most likely accounting for the fact that apples had a larger difference in pathogen reduction between surface and stem regions than oranges did. This observation is important because studies have shown that microorganisms can become enmeshed in cuticle regions, making removal difficult (Kenney et al. 2001). Other studies have reported that crevices, creases, pockets, and openings in skin, such as wounds and wounds, reduce accessibility of chlorine-based sanitizers and other sanitizers to pathogens embedded in such areas (Handbook of Fruits and Fruit Processing). Fatemi and Knabel (2006) also observed limited penetration into stem and calyx regions as evidenced by following NaI solutions using magnetic resonance imaging technology. These results indicate why sanitizer treatments completely inactivate microbes in crevices and creases of produce.
2.7 Factors Affecting Growth and Survival of *Listeria monocytogenes* on Fresh Produce

2.7.1 Atmospheric Gas Conditions and Temperature

It is widely known that various atmospheric gas conditions can influence the behavior of microorganisms. Some microorganisms grow well in the presence of low oxygen (microaerophiles), whereas other microorganisms grow well in presence of high oxygen. *L. monocytogenes* is facultatively anaerobic meaning it can survive and grow under both types of atmospheric conditions (Farber and Peterkin 1991). It is also known that storage temperature is an important factor affecting the growth of microorganisms, notably observed in ready to eat (RTU) vegetables (Francis and Beirne 2001). It is also known that although optimal growth of *L. monocytogenes* occurs at approximately 35°C (Jones and D’Orazio 2013), it is also capable of surviving and growing at refrigeration temperatures (Sinensky 1974). However it should be noted that reducing storage temperature will extend the lag phase and reduce the rate of growth of *L. monocytogenes* (Francis and Beirne 2001).

Berrang et al. (1989) conducted a storage study on fresh asparagus, cauliflower, and broccoli that showed growth of *L. monocytogenes* at 15°C after 8 days of storage in both controlled atmosphere storage and in air, although *L. monocytogenes* populations on broccoli and cauliflower were unchanged in both controlled atmosphere storage and in air stored at 4°C for up to 21 days (Berrang et 1989). Controlled atmosphere storage of produce occurs under lower oxygen and higher carbon dioxide concentrations than that which occurs in ambient air where the rate of respiration reduced and ripening is
slowed (Mitcham and Mitchell 2002). This study showed that temperature was a more important factor than gas composition with respect to changes in *L. monocytogenes*. It should be noted that in their experiment (Berrang et 1989), relative humidity conditions were not specified.

In another study, *L. monocytogenes* populations on packaged fresh cut vegetables were monitored during cold storage (Farber et al. 1997). Whole rutabagas, butternut squash, and onions, as well as packaged Caesar salad, carrots, coleslaw mix, and stir-fry vegetables were inoculated with *L. monocytogenes* and stored at 4 and 10°C for 9 days. *L. monocytogenes* population levels remained constant on all fresh-cut vegetables stored at 4°C for 9 days, except for carrots and butternut squash, where levels declined (no statistical significance was stated). Fresh-cut vegetables stored at 10°C, however, supported growth of *L. monocytogenes* on all vegetables tested (again no statistics given), except for chopped carrots, where the population decreased approximately 2 log units over a 9-day storage period (Farber et al. 1997). As was the case with the previous studies (Farber et al. 1997, Berrang et al. 1989), cold storage at 4°C can inhibited *L. monocytogenes* population growth over an extended period of time.

A study conducted by Francis and Beirne (2001) studied the effect of package atmosphere and storage temperature on growth and survival of *Escherichia coli* O157:H7 and *L. monocytogenes* on shredded lettuce, diced swedes (a cross between the cabbage and the turnip), dry coleslaw mix, soybean sprouts at storage temperatures of 4 and 8°C. Oxygen levels were approximately 20% at the beginning of storage and ranged from 0.5 to 3% at the end of the 12 day storage period. Carbon dioxide levels ranged from 10 to 25% at the end of storage. The authors did not mention relative
humidity conditions. *L. monocytogenes* populations remained constant on shredded lettuce, diced swedes, and on soybean sprouts but decreased on the dry coleslaw mix after 12 days storage at 4°C (Francis and O’Beirne 2001). As observed with the previous studies discussed, extended cold storage of fresh produce at 4°C effectively prevented *L. monocytogenes* growth. It is not clear from this study, however, if changes in gas composition played any role in microbial changes.

Another study by Beuchat and Brackett (1990) followed *L. monocytogenes* populations on whole and shredded carrots stored at 5 and 15°C over 24 days. Populations on whole carrots decreased to non-detectable levels after 24 days storage whereas levels increased on shredded carrots. The results of this study differ from that of the shredded lettuce results of Francis and O’Beirne 2001 in that slicing caused *L. monocytogenes* populations to increase. It is possible that the naturally occurring higher sugar concentrations in the carrots provided an adequate nutrient supply that allowed *L. monocytogenes* growth that normally would not occur on whole carrots during cold storage. However, the study confirms that *L. monocytogenes* population growth is restricted at colder storage temperatures compared to warmer ones (Beuchat and Brackett 1990).

**2.7.2 The Effect of Atmospheric Gas Conditions and Temperature on *Listeria monocytogenes* Behavior on Fresh Apples**

Sheng et al. (2017) studied the fate of *L. monocytogenes* on fresh apples stored at 1, 4, 10 and 22°C. Apples under the different storage treatments were sampled at 1, 4, 7 and 14-days for short-term storage at all four temperatures, and after 2, 4, 8, and
12 weeks at 1, 4, and 10°C which represents longer-term storage. During the 14 day storage experiment, \textit{L. monocytogenes} populations on Granny Smith apples at 1, 4, or 10°C C were reduced by 0.2–0.3 logs. During the 2-week short term storage, \textit{L. monocytogenes} population on Granny Smith apples stored for 14 days at 1, 4, or 10°C was reduced by up to 0.3 Log. At 22°C, there was up to a 1.2 Log10 CFU/apple reduction after the same storage time. During a longer term 12-week cold storage at 1, 4, and 10°C, \textit{L. monocytogenes} on Granny Smith apples decreased by up to 1.5 Log10 CFU/apple. Similar behavior occurred on stored conventional Granny Smith and Fuji apples. TPC and Y/M count remained unchanged for up to 12-weeks at all tested temperatures. This study demonstrated that although \textit{L. monocytogenes} did not grow on apples the small reductions that occurred cannot be relied upon as an intervention to eliminate \textit{L. monocytogenes} on contaminated fresh apples during long-term cold storage. One issue with this study is that there was no mention of the atmospheric conditions at which the apples were stored. This is significant because previous studies have shown that low humidity (53% relative humidity) can negatively affect \textit{L. monocytogenes} growth on lettuce leaves (Likotrafiti et al. 2013). Furthermore, Conway et al. (2000) showed that \textit{L. monocytogenes} populations increased on apple slices at both 10 and 20°C in air and in a controlled atmosphere (0.5% oxygen, 15% carbon dioxide). However, no growth was observed at 5°C under any of the atmospheric conditions (Conway et al. 2000). This finding is important because this temperature is close to the apple storage temperature of 4°C in packing houses (Simcox et al. 2001) and if one saw no growth on apple slices, one would probably not expect growth within
an apple wound, bruise, let alone an intact surface since there would be much less of a nutrient source for *L. monocytogenes* to grow.

The results from these two apple studies are similar to the results of the study by Berrang et al. (1989), where no significant *L. monocytogenes* population changes were observed during cold storage of any of the fresh produce they studied. Therefore, it seems likely that cold storage of produce (at 4 or 5°C) will not significantly reduce *L. monocytogenes* populations over an extended period of time.

### 2.7.3 Effect of Relative Humidity on *L. monocytogenes* Survival

Relative humidity is another factor influencing pathogen growth. Apple storage conditions commonly used can vary from controlled atmosphere settings with very high humidity, to ambient atmospheric conditions commonly observed in apple packing houses with moderate to high humidity of 86% and higher (Redfern et al. 2017, Simcox et al. 2001). A study done by Likotrafiti et al. (2013) on stored fresh lettuce leaves showed that low humidity (53% relative humidity) negatively affects *L. monocytogenes* growth, limiting increase to around 0.5 log cfu/cm² over a storage period of 7 days at 10°C. The study also showed that high humidity at low temperatures can promote survival of *L. monocytogenes*, as they reported no major change in *L. monocytogenes* populations over a 7 day holding period at 10°C at 90% relative humidity. Dreux et al. (2007) did a similar study looking at the effect of relative humidity on *L. monocytogenes* populations with parsley leaves. Their results were similar to the lettuce study done by Likotrafiti et al. (2013), as *L. monocytogenes* populations remained relatively constant at 100% RH over a 2 week storage period, but steadily declined at 45% relative humidity.
(Dreux et al. 2007). These results signify that high humidity can effectively preserve *L. monocytogenes* populations on fresh produce.

Redfern et al. (2017) also reported similar findings dealing with stainless steel surfaces. They reported that a larger percentage of viable cells recovered at 4°C than at 10 and 21°C in both high and medium humidity environments. They also reported that a larger percentage of viable cells was recovered under a higher humidity, lower temperature environment compared to a lower humidity, higher temperature environment (Redfern et al. 2017). These results are interesting since the temperature and humidity conditions are similar to that found in packing houses (albeit the packing houses have a 4°C holding temperature as opposed to a 10°C holding temperature) (Simcox et al. 2001). After observing the results with lettuce leaves, parsley, and stainless steel, it is possible that similar results occur on fresh apple surfaces.

It is clear that there is a history of *L. monocytogenes* contamination on fresh apples. A question that might arise is why would one have interest in studying the effects of storage conditions and sanitizers on *Listeria monocytogenes* populations on apples. The reason behind this is that storage is a unit operation in the apple packing process where *L. monocytogenes* levels could change over time, depending on the conditions present. Therefore, it is important to study the fate of *L. monocytogenes* under storage conditions. The addition of sanitizers to dump, flume, and wash water is considered a best practice. Therefore, studying the fate of *L. monocytogenes* during this process step is important to maximize pathogen reduction on fresh apples.
Given this information, this thesis will investigate potential interventions in the apple packing process that may prevent growth or results in decrease in populations of *Listeria monocytogenes*. In addition, the effects that fruit coatings, a coating antimicrobial emulsion, an aqueous antimicrobial solution, the presence of wounds, and differences in storage temperature and storage relative humidity might have on the growth and survival of *L. monocytogenes* during long term storage will be investigated. Finally, the effect that commercial sanitizers might have on *L. monocytogenes* populations on fresh apples in a simulated dump tank and the extent to which cross contamination of *L. monocytogenes* can occur will be investigated.

In the chapter that follows, the effects of two commercial fruit coatings on the fate of *Listeria monocytogenes* will be investigated.
CHAPTER 3:

DETERMINATION OF IN VITRO AND IN SITU FRUIT COATING EFFECTS ON

*Listeria monocytogenes*

3.1. Abstract

A plate overlay assay was performed to determine if commercially available carnauba wax or shellac fruit coatings had any effect on growth of *Listeria monocytogenes*. For each coating, dried disks were prepared by thoroughly filling the solutions into plastic molds to eliminate any possible antimicrobial effects of carrier solvents in the coating formulations. For each type of coating, zones of inhibition or accelerated population growth surrounding the dried disks were not observed after 24 and 48 hours incubation. This finding indicates that these coatings likely have no effects on *L. monocytogenes* growth on apples.

A storage experiment was also performed to study the fate of *L. monocytogenes* on inoculated apples during storage for up to 28 days. Variables tested were the application of carnauba or shellac coatings with or without lauric arginate or application of aqueous lauric arginate. During the 28 days of storage at 4°C, significant (p=0.05) decreases in *L. monocytogenes* were observed for all treatments and the control. After 28 days, no significant (p=0.05) population differences were observed between the carnauba or shellac coating and neither was significantly different from the control. Incorporation of 10% lauric arginate in either coating formulation significantly (p=0.05) but only slightly decreased *L. monocytogenes* populations on intact inoculation sites,
but not on the wounded sites. Aqueous 10% lauric arginate did not result in a greater reductions compared to the incorporation of lauric arginate into the fruit coatings.

3.2. Introduction

Edible coatings are commonly applied to fresh produce, as well as other food products. Two commonly used coatings applied to fresh produce and other food products are carnauba wax and shellac (Goncalves et al. 2010). Coatings act as protective films on produce by raising carbon dioxide levels and lowering oxygen levels. This coating reduces the metabolic activity of the fruit and increases resistance to plant pathogens, thus increasing fruit storage life (Goncalves et al. 2010). However, the question that needs to be answered is: Do commonly used fruit coatings have effects against growth of *L. monocytogenes* and survival? This finding is important and potentially useful because coatings could serve as an alternative method for reducing the risk of *L. monocytogenes* on apples. One potential advantage of using coatings, such as carnauba wax and shellac, is that they can be easily sprayed onto fresh produce where they adhere for longer periods of time than would occur using aqueous sanitizer solutions. This approach would allow one to use a lower treatment volume since a relatively small volume of fruit coating (20-25 mL) is needed to completely coat an apple. Additionally, waxes provide an effective humidity barrier for fresh produce (Bourtoom 2008), thus slowing water loss and effectively preserving fruits for extended periods of time (Bai and Krochta 1994).

The first purpose in this study was to perform a plate overlay assay to determine if commercially available carnauba wax and shellac formulations have any effects on
the growth of *Listeria monocytogenes*. Although *L. monocytogenes* growth conditions present during the plate overlay assay differ from those present on a contaminated apple, observing any effects from either carnauba wax or shellac would provide a reason to further pursue this approach toward reducing risks. After performing the plate overlay assay, an apple storage experiment was performed to look at the effects of coating treatments, a coating antimicrobial emulsion, and an aqueous antimicrobial solution on *L. monocytogenes* populations during storage for up to 28 days.

### 3.3. Materials and Methods

#### 3.3.1. Preparation of Wax and Shellac Disks

Flexible round silicone candy mold wells (26 X 6 mm, BakeDeco, Brooklyn, NY) were filled to capacity with Prima Fresh® P3000 carnauba wax or Prima Fresh® P450 sterile shellac fruit coating formulations (Pace International, Wapato, Washington). The filled molds were held overnight at 37°C in a temperature-controlled incubator (Model 2300, Sheldon Manufacturing Inc., Cornelius, OR). Each morning, the evaporated solvents were replaced with additional coating formulation and returned to the incubator. This procedure was repeated for 4 additional days with successively smaller volumes of the coatings until the overnight reduction approached zero. Final solidification of the discs was achieved by holding the molds overnight at 55°C. The solid disks were removed from the molds and stored in an airtight jar for 4 weeks before use.

#### 3.3.2. Strain Selection and Preparation of Working Cultures

*L. monocytogenes* isolates F2365, 1/2a 35D, 1/2a 23A, 19-P-14M, and Scott A were obtained from a culture collection in the Food Microbiology Laboratory at Penn...
State University. Each isolate was grown up in tryptic soy broth w/yeast extract (TSBYE; Difco Laboratories, Detroit, MI) as described by D’Orazio et al. 2013 and incubated overnight for 24 hours at 35.5°C. The optical density for each strain was adjusted to 0.5 AU to produce a concentration of $10^9$ CFU/mL. The individual isolates were combined into a single working culture.

### 3.3.3. Preparation of Inoculum

Thirty 1-ml centrifuge tubes were each filled with 1 mL of the combined working culture. Each tube was centrifuged for 3 minutes at 10000 x g, after which, the supernatant was decanted and replaced with 1 mL of 0.85% sodium chloride (NaCl) and then spun down again. The wash process was repeated once. The washed mixtures were then vortexed to evenly distribute *L. monocytogenes* cells in the tube after which the contents of all the individual tubes were combined in a beaker. The concentration of *L. monocytogenes* in the inoculum was approximately $10^8$ CFU/mL. The inoculum was subsequently diluted to produce a *L. monocytogenes* concentration of $10^5$ CFU/mL. Actual initial inoculum levels were determined by plating before each experiment was conducted.

### 3.3.4. Preparation of the Overlay

The plate overlay assay method described by Cutter (1999) was used. Eight mL of TSA was poured into multiple petri dishes. Dried carnauba wax disks (or shellac disks) prepared as specified above, were aseptically placed onto the center of the plate. Semisoft TSA was prepared, autoclaved, and cooled to 47°C in a water bath. A *L. monocytogenes* overnight culture was grown up as described in the protocol by Jones and D’Orazio (2013). Ninety-six µL of the culture was added to 12 mL of the semisoft
TSA previously cooled to 47°C. This temperature was as cool enough for *Listeria* cells to remain alive, but also warm enough for the semisoft TSA to not solidify. Twelve mL of this semisoft TSA/overnight culture mix was poured into each plate. Plates were incubated at 37°C overnight for 24 hours and the overlay assay was replicated twice.

3.3.5. Preparation and Inoculation of Apples

Red Delicious apples were obtained from a local apple grower. Wounds were made using sterile cork borer (5 mm inside diameter) to a depth of 2 mm. Three replicate apples were spot inoculated by placing a single 10 uL drop of inoculum on each shoulder site. Inoculated apples were then placed on a plastic weighing boat for air drying for 1 hour at room temperature. The inoculated apples were randomized before coating treatments were applied.

3.3.6. Coatings and Treatments

For the storage experiment, commercially available carnauba wax (P3000, PACE International, Wapato, Washington) and shellac (P450 PACE International, Wapato, Washington) fruit coatings were used. Inoculated Red Delicious apples were coated with carnauba wax, shellac, a carnauba wax-lauric arginate mixture (10%), a shellac-lauric arginate mixture (10%), or an aqueous lauric arginate solution (10%). Both the carnauba wax-lauric arginate and shellac-lauric arginate mixtures were prepared by dissolving 50 grams of lauric arginate powder into 10 mL ethanol which was subsequently added to 490 mL of the desired coating and emulsified using a homogenizer (Marshall Scientific, Hampton, NH). Alternatively, an aqueous solution of lauric arginate was prepared by dissolving 50 grams of lauric arginate powder 10 mL ethanol which was subsequently added to 490 mL distilled water. The final lauric
arginate concentration was 10,000 ppm (10%). Untreated inoculated apples rinsed with distilled water and subsequently dried served as controls. The apples were aseptically coated using a spray bottle (25 full sprays for 20 mL) and then allowed to dry at room temperature for 5 minutes in plastic weighing boats.

3.3.7. Storage Conditions

In Experiment 1, the inoculated and coated or treated apples were held in a temperature controlled refrigerated incubator at 4°C for up to 1, 7, 14, 21, and 28 days. Immediately after coating, and at the end of each storage interval, *L. monocytogenes* populations were determined. Relative humidity was not actively monitored during the experiment but was later determined using a portable CO₂, thermos-hygrometer (Therm-Hydro-CO₂, model 98132S, MIC, Taiwan) to be 33% under the conditions of the experiment.

In Experiment 2, the inoculated and coated apples were held at 4°C in a temperature controlled refrigerated incubator or at 20°C under ambient conditions within the Penn State Food Safety Pilot Plant. At 4°C, two relative humidity conditions were maintained; 50% and 90%. The higher humidity was maintained by storing the apples in plastic container in which a damp fabric was placed on the bottom. Wire racks were placed on top of the fabric to assure that free moisture did not contact the apples. The apples were prevented from contacting themselves or moving out of place by placing them in molded pressed paper fiber trays normally used in commercial packing operations. The lower humidity level was achieved by storing apples on the paper trays
placed within the incubator but outside of the plastic box. At 20°C, apples were stored on paper trays under a biosafety hood in the food safety pilot plant.

Relative humidity (RH), Oxygen (O2), and Temperature were measured using a portable meter (Therm-Hygro-CO2, model 98132S, MIC, Taiwan). Oxygen gas was measured with a portable monitor (Pro GasBadge, Industrial Scientific, Pittsburg, PA). Ethylene gas was measured using a AP-20 aspiration pump and ethylene detection tubes (No. 108B, 0.1-100 ppm) from Kitagawa (Komyo Rikagaku Kogyo, Japan).

3.3.8. *Listeria monocytogenes* Enumeration

After each treatment, a sterile cork borer and a sterile knife were used to remove plugs to a depth of 10 mm from the wounded and intact sites of each apple. Plug weights varied between 1.0 and 1.2 grams. The separated wounded and intact plugs from 3 apples were added together to 27.0 ml of sterile 0.85% NaCl in 13 oz (384 mL) WhirlPack® bags. The bags were placed in a stomacher (SPW Industrial, Laguna Niguel, CA) for homogenization at 60 rpm for 2 minutes. After homogenization, 3 ml of the homogenate were mixed with 27 ml of 0.85% NaCl and serially diluted (10⁻¹, 10⁻², and 10⁻³) for plating (100 µL) on modified Oxford agar (MOX). This process was repeated once, allowing for two sets of three plugs. Each plug was 1 cm³ in volume. Plates were incubated for 20 hours at 35.5°C. Populations were determined by dividing the number of visible colonies by 3 and subsequently multiplying by the dilution factor, given that there were three plugs being homogenized at a time and each plug was 1 cm³ in volume. Final populations were in units of log CFU/cm³.
3.3.9. Experimental Design and Statistical Analysis

Recovered *L. monocytogenes* population values were analyzed using 2-way ANOVA. The significance of treatments was determined at p<0.05. Immediate population reductions were calculated by taking the difference between the non-treated apple populations at time zero and the treated apple populations at time zero. Longer-term 28-day population reductions were calculated by taking the difference between treated apple populations at 28 days and non-treated apple populations at time zero. At each sampling point, 3 apples were randomly sampled from each batch (n=18). Results were presented as mean ± SD (standard deviation).

3.4 Results and Discussion

3.4.1. Plate Overlay Assay

Figures 3.1 and 3.2 show the results of the assay after incubation at 37°C for 24 and 48 hours. Overlay assay plates for carnauba wax appeared different from the overlay assay plates for shellac because each set of plates was done at a separate time and at a different bacterial concentration. For all plates observed, no zones of inhibition present or areas of accelerated growth were observed. Darkening observed around wax is related to the color of the wax and not pathogen inhibition. A weakness of this study is the variability in the quality of the *L. monocytogenes* lawn despite efforts to maximize the microbial concentration in a quest to obtain consistently small and numerous colonizes. Further work may need to go into optimizing this assay. Nevertheless, it can be concluded that there are no effects of carnauba wax or shellac formulations on the growth of *L. monocytogenes*. 
These results are in contrast with the findings of Jo et al. (2014) who observed population reductions of both *L. monocytogenes* and *E. coli* O157:H7 inoculated into tubes containing a liquid carnauba wax formulation. *L. monocytogenes* reductions of 1 log were observed after 24 hours storage. It should be noted however, that when the researchers performed this study, the wax formulation contained solvents (ethanol and other volatiles). It has been well documented that the certain solvents, such as alcohols, can bring about a reduction in *L. monocytogenes* populations (Park et al. 2011, Rodgers et al. 2004). It is therefore possible that the presence of solvents in the wax led to the population reduction observed by Jo et al. (2014). In this plate overlay assay, any solvents present in the formation were evaporated. Separating carnauba wax and shellac constituents from organic solvents may have eliminated a possible solvent based anti-*Listeria* effect. In the study by Jo et al. (2014) it was not clear if the observed 1 log reduction could be attributed to carnauba wax or shellac, or from the solvents it was mixed in. The results from this chapter suggest that solvents may have played a role in the reduction. Nevertheless, the overlay assay cannot be definitively applied to the case of whole apples and a further study using whole apples is warranted.

3.4.2. Apple Storage Study

In this section *L. monocytogenes* was inoculated onto whole intact and wounded apples and any effects of coating formulations on microbial levels were examined.

3.4.2.1. *L. monocytogenes* Population Changes on Intact Apples as Affected by Commercial Fruit Coatings.
Figures 4.1A and Table 4.1 show population changes on intact apples before and after application of carnauba wax and shellac fruit coatings and during storage for up to 28 days at 4°C. Immediately after inoculation, *L. monocytogenes* levels were 4.57 log CFU/cm². Upon initial application of the coating treatments, a slight but insignificant (0.16 log) reduction of *L. monocytogenes* occurred, presumably due to the carrier solvents. There were no significant differences in *L. monocytogenes* populations observed at each storage interval between the control and either of the carnauba wax or shellac treatments (p=0.05). Populations remained unchanged for the first 7 days of storage and then declined for the next 14 days (p=0.05), until leveling off for the final 7 days of the study. Total reductions in *L. monocytogenes* populations ranged between 1.67 and 1.88 logs.

The reductions observed for carnauba wax fell short of those observed by Kenney et al. (2002), who looked at *S. enterica* and *E. coli* O157:H7 population reductions on fresh apples coated with carnauba wax over a period of 6 weeks at 2°C. In their study, population reductions for *S. enterica* ranged from 3.59 to 4.25 log\(^{10}\) CFU/apple after 3 weeks and 2.50 to 2.79 log CFU/apple after 6 weeks. Population reductions for *E. coli* O157:H7 ranged from 1.32 to 3.05 log CFU/apple after 3 weeks and 4.16 to 4.95 log CFU/apple after 6 weeks (Kenney et al. 2002). One can speculate that the reason for the discrepancy in observations could be attributed to differences in cell wall structure given that *E. coli* and *S. enterica* are Gram-negative bacteria while *L. monocytogenes* is Gram-positive. However, there are no studies to support this. It should also be noted that relative humidity values were not mentioned by Kenney et al. (2002).
In addition, the *L. monocytogenes* reductions observed in the present study fell far below the reported initial reductions for carnauba wax of 1.98 log CFU/g reported by Jo et al. (2014). It should be noted, however, that in their study, a carnauba wax formulation that likely contained volatile solvents with antimicrobial ability was used. Also, the model system used did not account for possible coating-fruit interactions.

3.4.2.2. *L. monocytogenes* Population Changes on Wounded Apples as Affected by Commercial Fruit Coatings.

Figure 4.1B and Table 4.2 show *L. monocytogenes* population changes on apple wound sites treated or not treated with carnauba wax and shellac fruit coatings. Immediately after inoculation, *L. monocytogenes* levels were 4.76 log CFU/cm². Initial population reductions upon application of carnauba wax or shellac were only (0.20 logs). Thereafter, population changes followed the same pattern as the intact apples. Total reductions ranged from 1.72 to 1.87 logs. These findings illustrate that puncture wounds on plain fruit coatings do not present additional risk for *Listeria monocytogenes* growth. Coatings, however, did not lead to lower *L. monocytogenes* populations recovered compared to the control.

3.4.2.3. *L. monocytogenes* Population Changes on Intact Apples Treated with Lauric Arginate Incorporated into Fruit Coatings or Aqueous Lauric Arginate.

Figure 4.2A and Table 4.1 show population changes on intact apples treated with carnauba wax and shellac fruit coatings incorporated with lauric arginate (10% w/v), aqueous lauric arginate (10% w/v), and compared them to those of the control. Immediately after inoculation, *L. monocytogenes* levels were 4.57 log CFU/cm². For both coating-antimicrobial treatments tested, *L. monocytogenes* populations significantly
declined upon application (p=0.05) as expected although this was not observed with either the aqueous lauric arginate treatment or the control that contained no solvents.

Populations remained unchanged for the first 7 days of storage and then declined for the next 14 days (p=0.05), until leveling off for the final 7 days of the study. Also similar was the slightly greater effect observed for the coatings containing lauric arginate compared to the aqueous lauric arginate-treated apples or the control (p=0.05) with total reductions as high as 2.24 logs. Log reductions at 28 days for aqueous lauric arginate (1.78 log CFU/cm³) were not significantly different than the control (1.67 log CFU/cm³). Population reductions observed fell far short of the reported 5 or greater log reductions reported by Taormina and Dorsa (2009) when they applied 5 and 9.1% aqueous lauric arginate to fresh hams (Taormina and Dorsa 2009). However, they used a different food product, possibly leading to different interactions than that on the surface of a fresh apple. It is possible that the greater effect of lauric arginate in the coatings, as compared to the aqueous solution, was due to synergistic effects of the coating and the antimicrobial. Lauric arginate acts on the cytoplasmic membrane of L. monocytogenes, disrupting the proton motive force within the membrane.

**3.4.2.4. L. monocytogenes Population Changes on Wounded Apples Treated With Lauric Arginate Incorporated into Fruit Coatings or Aqueous Lauric Arginate.**

Figure 4.2B and Table 4.2 show population changes on wounded apples treated with carnauba wax and shellac fruit coatings incorporated with lauric arginate (10% w/v), aqueous lauric arginate (10% w/v), and compared them to those of the control. Immediately after inoculation, *L. monocytogenes* levels were 4.76 log CFU/cm³. Similar to intact apples, initial population reductions for the coating with lauric arginate
treatments were significant (p=0.05) but small which was not observed with either the aqueous lauric arginate treatment or the control. Population changes followed the same pattern as the intact apples. However, population reductions observed for wounded apples treated with lauric arginate incorporated into fruit coatings and aqueous lauric arginate were not significantly differed from the control (p=0.05).

3.5 Conclusions

Based on the results of the plate overlay assay, neither carnauba wax nor shellac coatings appear to possess any anti- or pro-Listeria activity, as evidenced by a lack of clearing zones or signs of enhanced growth observed around the disks on the plate overlay assay. While no activity was observed from the plate overlay assay, it was necessary to test carnauba wax and shellac coatings on L. monocytogenes populations on whole apples, due to the fact that the pathogen might behave differently on the surface of apples (in situ) compared to the plate overlay system (in vitro). In addition, the extent to which fruit coatings emulsified with an antimicrobial would provide any enhanced anti-Listeria effects was compared to coatings or the antimicrobial alone.

Experimental findings showed that the presence of puncture wounds inhibit the effectiveness of coating-antimicrobial treatments, but not pure fruit coatings or aqueous lauric arginate. A possible explanation for such findings is facilitated pathogen growth within wounded apples. Considering that the storage study lasted 28 days, it is possible that the extended storage time period allowed for a much stronger penetration and attachment of pathogen cells that did not occur on intact apples, leading to a decline in the effectiveness of coating-antimicrobial treatments.
Figure 3.1a. Plate overlay assay of dried shellac fruit coating on a *L. monocytogenes* lawn after 24 hours at 37°C.

Figure 3.1b. Plate overlay assay of dried shellac fruit coating on a *L. monocytogenes* lawn taken after 48 hours at 37°C.
**Figure 3.2a.** Plate overlay assay of dried carnauba wax fruit coating on a *L. monocytogenes* lawn after 24 hours at 37°C.

**Figure 3.2b.** Plate overlay assay of dried carnauba wax fruit coating on a *L. monocytogenes* lawn after 48 hours at 37°C.
Figure 3.3. *Listeria monocytogenes* populations on A) intact and B) wounded apples coated or not coated with carnauba wax or shellac stored for up to 28 days at 4° C. Mean ± SD, n=18.
Figure 3.4. *Listeria monocytogenes* populations on A) intact and B) wounded apples coated with carnauba wax or shellac with and without added lauric arginate and stored for up to 28 days at 4° C. Mean ± SD, n=18.
Table 3.1. *Listeria monocytogenes* populations on intact apples held at 4°C and 35% relative humidity, treated with carnauba wax, shellac, carnauba wax + lauric arginate, shellac + lauric arginate, aqueous lauric arginate, and no treatment.

*Listeria monocytogenes* (log CFU/cm$^3$) ± SD

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<th>Storage time (days)</th>
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<th>Carnauba wax</th>
<th>Shellac</th>
<th>10% LA in Carnauba wax</th>
<th>10% LA in Shellac</th>
<th>10% LA (aq.)</th>
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<td>0</td>
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<td>3.53 ± 0.08Cc (-1.04)</td>
<td>3.06 ± 0.14Dd (-1.51)</td>
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<td>2.50 ± 0.07Ef (-2.07)</td>
<td>2.79 ± 0.01De (-1.78)</td>
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Values within rows marked with different capital letters are significantly different from each other (p=0.05). Values within columns marked with different lowercase letters are significantly different from each other (p=0.05). Values in parentheses at each time interval are population changes from un-treated apples measured before storage. Mean ± SD, n=18.
Table 3.2. *Listeria monocytogenes* populations on wounded apples held at 4°C and 35% relative humidity, treated with carnauba wax, shellac, carnauba wax + lauric arginate, shellac + lauric arginate, aqueous lauric arginate, and no treatment.

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<th>Treatment</th>
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<td>No Treatment</td>
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<td>10% LA in Carnauba wax</td>
<td>4.32 ± 0.06Bb</td>
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<td>10% LA (aq.)</td>
<td>4.37 ± 0.03Bb</td>
</tr>
<tr>
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<td>(-0.39)</td>
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</table>

Values within rows marked with different capital letters are significantly different from each other (p=0.05). Values within columns marked with different lowercase letters are significantly different from each other (p=0.05). Values in parentheses at each time interval are population changes from un-treated apples measured before storage. Mean ± SD, n=18.
CHAPTER 4:
FATE OF *LISTERIA MONOCYTOGENES* ON UNCOATED APPLES DURING STORAGE AT DIFFERENT TEMPERATURE AND RELATIVE HUMIDITY LEVELS

4.1 Abstract

The effects of storage temperature (4 or 20°C), storage relative humidity (50 or 89 % RH), apple variety (Fuji or McIntosh), and intact or wounded inoculations sites on *L. monocytogenes* populations were determined. With the exception of intact Fuji apples stored at 20°C and 50% RH, no significant *L. monocytogenes* populations reductions were observed for any of the temperature and relative humidity combinations over 28 days. Furthermore, there were not any differences between the two varieties, despite differences in tissue pH, titratable acidity, and total soluble solids. The location of the inoculation site did not have significant effects on *L. monocytogenes* populations, except for wounded apples at 14 and 21 days storage at 20°C and 50% RH where approximately 1 log growth was observed. Insignificant differences (p=0.05) between populations recovered on MOX and TAL plates for all storage temperature and humidity conditions indicate that cell injury did not overestimate *L. monocytogenes* counts, showing that the *L. monocytogenes* populations recovered were predominantly viable cells.
4.2 Introduction

It is well known that storage temperature strongly influences the growth of foodborne pathogens, with *L. monocytogenes* being no different. Optimal growth of *L. monocytogenes* occurs at approximately 35°C (Jones and D’Orazio 2013). However, the pathogen also has psychrophilic properties that enable it to survive and grow at refrigeration temperatures (Sinensky 1974). This observation is due to its ability to modify membrane fatty acid composition from the iso- to the anteiso- form which results in greater membrane fluidity at lower temperatures, when compared to mesophilic bacteria in which decreased membrane fluidity at lower temperatures slows growth (Wilkinson et al. 2015). Although *L. monocytogenes* is capable of surviving and growing at low temperatures, refrigeration extends its lag phase and reduces growth rate (Francis and Beirne 2001), thus preventing it from reaching its maximum growth potential. Humidity is also known to affect the growth of *L. monocytogenes*. Inoculation studies conducted on stainless steel (Redfern 2017) or fresh produce surfaces (Likotrafiti et al. 2013, Dreux et al 2007) have demonstrated that low humidity conditions negatively affect survival of *L. monocytogenes*, whereas high humidity conditions enhance survival.

Recent studies have demonstrated that under certain conditions, *L. monocytogenes* can grow on apples. Glass et al. (2015) looked at the growth of *Listeria monocytogenes* in apples punctured with sticks prior to coating with caramel. *L monocytogenes* populations increased more than 3 log CFU in the punctured caramel apples, compared to only 1 log CFU in non-punctured or coated apples after 3 days at 25°C (Glass et al. 2015). The authors stated that the growth promoting environment
was made possible through the puncturing process which released cell contents (juice) while the caramel provided a nutrient source while raising the pH. A 2016 study by Salazar et al. (2016) showed that when *L. monocytogenes* was inoculated onto fresh Granny Smith and Gala apples at 7 log CFU/mL and then stored for 15 days at 5°C, the pathogen survived, but did not grow. However, on apples punctured with a stick, and then coated with caramel, inoculated *L. monocytogenes* grew by approximately 3 log CFU/mL within 3 days of storage under the same conditions. The same study (Salazar et al. 2016) also showed that when the inoculum was allowed to dry for 24 h at 5°C, growth was significantly slowed compared to drying for 2 h at 25°C, illustrating that low temperature slows the growth of *L. monocytogenes* on apples.

A more recent study by Sheng et al. 2017 reported no significant population changes in inoculated *L. monocytogenes* populations on whole intact Granny Smith and Fuji apples stored for up to 4 weeks at 1, 4, 10, and 22°C. Humidity conditions were not specified. Based on the work that has been done to date, there is currently inadequate information on relative humidity levels affects *L. monocytogenes* populations on fresh apples at various storage temperatures, as well as how edible coatings impact *L. monocytogenes* populations on fresh apples.

Apples and other fresh produce can become bruised, punctured, or otherwise wounded during handling and transport on the packing line. Such wounding might facilitate the growth of *L. monocytogenes* on fresh apples under certain circumstances. However, there are no studies that have compared *L. monocytogenes* survival and growth on intact and wounded whole apples.
In this chapter, the effects of storage temperature and relative humidity on *L. monocytogenes* populations during apple storage were studied at relative humidity values of 50% or 89% at 4°C as well as at a relative humidity of 50% at 20°C. No coatings will be applied since the previous chapter showed that they do not have significant pro- or antimicrobial activity. A thin agar layer (TAL) method was used to account for any injured *L. monocytogenes* cells recovered during the storage process since it is possible that the selective MOX media may limit total recovery of living cells. Comparing populations recovery by the TAL and MOX methods will determine whether the *L. monocytogenes* populations recovered were predominantly viable or injured cells.

4.3 Materials and Methods

4.3.1. Strain Selection and Preparation of Working Cultures

*L. monocytogenes* isolates F2365, 1/2a 35D, 1/2a 23A, 19-P-14M, and Scott A were obtained from a culture collection in the Food Microbiology Laboratory at Penn State University (all coming from a nearby packing house). Each isolate was grown up in tryptic soy broth with yeast extract (TSBYE; Difco Laboratories, Detroit, MI) as described by D’Orazio et al. (2013) and incubated overnight for 24 hours at 35.5°C. The optical density for each strain was adjusted to 0.5 AU to produce a concentration of $10^9$ CFU/mL (plated out). The individual isolates were combined equally into a single working culture.

4.3.2. Preparation of Inoculum

Thirty 1-ml centrifuge tubes were each filled with 1 mL of the combined working culture. Each tube was centrifuged for 3 minutes at 10000 x g, after which, the supernatant was decanted and replaced with 1 mL of 0.85% sodium chloride (NaCl),
vortexed, and then spun down again. The wash process was repeated once. The washed mixtures were then vortexed to evenly distribute *L. monocytogenes* cells in the tube after which the contents of all the individual tubes were combined in a beaker. The concentration of *L. monocytogenes* in the inoculum was approximately 10^8 CFU/mL (plated out). The inoculum was subsequently diluted in 0.85% NaCl to produce a *L. monocytogenes* concentration of 10^7 CFU/mL. Actual initial inoculum levels were determined by plating before each experiment was conducted.

4.3.3. Preparation and Inoculation of Apples

Unwaxed Fuji and Gala apples were obtained from a local grower. One shoulder of each apple was wounded; the other shoulder was left intact. Wounds were made using sterile cork borer (5 mm inside diameter) to remove a small section of the apple at a depth of 2 mm. Three replicate apples were spot-inoculated by placing a single 10 μL drop of inoculum on each shoulder site, resulting in a *L. monocytogenes* concentration of 10^5 CFU/mL on each inoculated site. Inoculated apples were then placed on a plastic weighing boat for air drying for 1 hour at room temperature. The inoculated apples were randomized before treatments were applied. No antimicrobials or coating treatments were used in this study.

4.3.4. Storage Conditions

Inoculated apples were held at 4°C in a temperature controlled refrigerated incubator at 50% and 90% relative humidity at 20°C in the Penn State BSL2 Food Safety Pilot Plant. The higher humidity was maintained by storing the apples in plastic container in which a damp absorbent fabric was placed on the bottom of the container.
Wire racks were placed on top of the fabric to assure that free moisture did not contact the apples. The apples were prevented from contacting themselves or moving out of place by placing them in molded pressed sterilized paper fiber trays normally used in commercial packing operations. The container was covered with a plastic lid to allow humidity conditions to reach a steady equilibrium value. The lower humidity level was achieved by storing apples on the paper trays placed within the incubator but outside of the plastic box. At 20°C, apples were stored on paper trays under a biosafety hood in the food safety pilot plant.

Relative humidity (RH), oxygen (O2), and temperature were measured initially and at each sampling period using a portable multi-meter (Therm-Hygro-CO2, model 98132S, MIC, Taiwan). Oxygen gas was measured with a portable meter (Pro GasBadge, Industrial Scientific, Pittsburg, PA). Ethylene gas was measured using an AP-20 aspiration pump and ethylene detection tubes (No. 108B, 0.1-100 ppm) from Kitagawa (Komyo Rikagaku Kogyo, Japan).

4.3.5. *Listeria monocytogenes* Enumeration

After each treatment, a sterile cork borer and a sterile knife were used to remove plugs to a depth of 10 mm from the wounded and intact sites of each of the 3 apples. Plug weights varied between 1.0 and 1.2 grams. The separated wounded and intact plugs from 3 apples were added together to 27.0 ml of sterile 0.85% NaCl in 13 oz (384 mL) WhirlPack® bags. The bags were placed in a stomacher (SPW Industrial, Laguna Niguel, CA) for homogenization at 60 rpm for 2 minutes. After homogenization, 3 ml of the homogenate were mixed with 27 ml of 0.85% NaCl and serially diluted (10\(^{-1}\), 10\(^{-2}\),
and $10^{-3}$) for plating (100 µL) on modified Oxford agar (MOX). This process was repeated once, allowing for two sets of three plugs. Each plug was 1 cm$^3$ in volume. Plates were incubated for 20 hours at 35.5°C. Populations were determined by dividing the number of visible colonies by 3 and subsequently multiplying by the dilution factor, given that there were three plugs being homogenized at a time and each plug was 1 cm$^3$ in volume. Final populations were in units of log CFU/cm$^3$.

4.3.6. Thin Agar Layer (TAL) Plate Enumeration

The thin agar layer (TAL) method of Kang and Fung (1999) was used to estimate the extent to which *L. monocytogenes* counts might have been underestimated due to their failure to grow on MOX selective media. TAL plates were prepared by pouring 25 mL sterilized modified Oxford (MOX) agar in a Petri dish and adding two subsequent layers of melted tryptic soy agar (TSA) with each layer being 2.5 mL. MOX agar selects for *L. monocytogenes*, while TSA allows for recovery of injured cells. After complete solidification of media, 100 µL of inoculum was spread plated on top of each TAL plate.

The same method described in section 4.3.6 was used for extraction and enumeration of *L. monocytogenes* except that serial dilutions ($10^{-1}$, $10^{-2}$, and $10^{-3}$) were plated (100 µL) on the TAL plates. This process was repeated once. Plates were incubated for 20 hours at 35.5°C. An estimate of the extent of cell injury was determined by comparing cell populations determined on MOX versus the TAL.

4.3.7. Experimental Design and Statistical Analysis

Recovered *L. monocytogenes* population values were analyzed using 2-way ANOVA. The significance of treatments was determined at $p<0.05$. Immediate
population reductions were calculated by taking the difference between the non-treated apple populations at time zero and the treated apple populations at time zero. Longer-term 28-day population reductions were calculated by taking the difference between treated apple populations at 28 days and non-treated apple populations at time zero. At each sampling point, 3 apples were randomly sampled from each batch (n=18). Results were presented as mean ± SD (standard deviation).

4.4. Results and Discussion

4.4.1. L. monocytogenes Population Changes on Intact Apples during Storage.

Tables 4.3, 4.4, and 4.5 show that immediately after inoculation, McIntosh apples exhibited an average (n=3) L. monocytogenes population of 4.30 ± 0.11 log CFU/cm³ while the level on Fuji apples was 4.49 ± 0.14 log CFU/cm³.

Figures 4.3 and 4.4 show that L. monocytogenes populations on intact apples at each temperature and relative humidity conditions were statistically the same (p=0.05) between 0 and 28 days, except for intact Fuji apples stored at 20°C and 50% RH. Only intact Fuji apples stored at 20°C and 50% RH showed a slight, but significant (p=0.05) population decline (about 1 log CFU/cm³), over the 28 days storage period (Figure 4.4).

Results were similar to those obtained by Sheng et al. (2017), who observed stable populations on intact apples stored for up to 4 weeks at 4°C. It should be noted, however, that they only studied apples stored at 1, 4, and 10°C over a period of 12 weeks, and at 22°C over a period of 2 weeks. Also, their study did not mention the relative humidity conditions that they used (Sheng et al. 2017).
In the current study, an interesting point is that humidity at 4°C was not a significant (p=0.05) effect over the course of the 28-day storage period. Dreux et al. (2007) and Zoz et al. (2016) observed greater *Listeria monocytogenes* reductions on parsley and polypropylene coupons, respectively, at lower relative humidity conditions. Our experimental results are unexpected, given that low relative humidity conditions are known to restrict *L. monocytogenes* growth and high relative humidity conditions are known to stimulate *L. monocytogenes* growth (Zoz et al. 2016).

4.4.2. *L. monocytogenes* Population Changes on Wounded Apples during Storage.

Figures 4.5 and 4.6 show that after the 28-day storage period, with the exception of wounded McIntosh apples stored at 4°C and 50% RH for wounded apples tested at each temperature and humidity condition, *L. monocytogenes* populations were statistically the same (p=0.05) as those at the beginning of the storage study. Wounded apples of both varieties stored at 20°C at 50% humidity displayed significant population increases by 14 days of storage (p=0.05), something that was not observed with other wounded apples at any of the other storage temperatures or humidity values. This observation could be due to a microenvironment with high amounts of available sugar by the wounds that, despite the acidic condition, permitted some *L. monocytogenes* growth. Although *L. monocytogenes* can grow at refrigeration temperatures, it is known to grow more rapidly at room temperature, given similar non-temperature growth conditions are present (Sinensky 1974). It should be noted that, despite the ability of *L. monocytogenes* to grow at low temperatures, reducing storage temperatures will extend the lag phase and reduce the overall amount of growth (Francis and O’Beirne 2001).
Glass et al. (2015) stated that wounded apples can release juice from apple cells, possibly accounting for the short-term increase in populations on wounded apples stored at 20°C, when compared to intact apples stored at the same temperature.

It is known that \textit{L. monocytogenes} possesses a functional arginine deiminase (ADI) system and analysis of deletion mutants reveals that it contributes to both growth and survival of the bacterium under acidic conditions (Ryan et al. 2008). Adaptation to low pH augments \textit{L. monocytogenes} resistance to an acidic environment, as \textit{L. monocytogenes} can survive at low pH after prior induction in such an environment (Phan-Thanh et al. 2000, Hill et al. 1995). The initial increase in \textit{L. monocytogenes} between 7 and 14 days as shown in Figures 4.5 and 4.6 was followed by decreases in populations between 14 and 28 days storage. This finding could be attributed to the depletion of apple sugars as a nutrient source for \textit{L. monocytogenes} during storage. The fact that no significant population decreases were observed for wounded McIntosh and intact Fuji apple stored at high humidity (89% RH) conditions studied (Figures 4.5 and 4.6) suggests that high humidity prolongs survival, as was shown by Redfern et al. (2017). They observed that high humidity led to a higher \textit{L. monocytogenes} population recovery than low humidity, albeit on stainless steel coupons. Therefore, the results in the current study suggest that a combination of elevated humidity (50% RH and higher) and elevated temperature (20°C) can prolong the survival of \textit{L. monocytogenes} populations on fresh apples during extended storage for up to 28 days.
4.4.3 Injured and Uninjured *Listeria monocytogenes* Cells and Background Microflora

Tables 4.12, 4.13, and 4.14 show recovered populations of *L. monocytogenes* on TSA, TAL, and MOX media plates at the different storage conditions tested. No significant differences (p=0.05) between populations recovered from MOX plates and TAL plates were observed, signifying that there were few injured *L. monocytogenes* cells present after the inoculation period at all storage temperatures and humidity conditions tested (p=0.05). Also, non-significant differences (p=0.05) between populations recovered from TAL plates and TSA plates signify that the amount of background microflora present on the apples were unaffected by storage temperature and relative humidity. Thus, competitive inhibition effects can be ruled as possible reasons for the storage behavior of *L. monocytogenes*.

4.4.4. Gas Concentration Observations

Table 4.15 reports the observed gas conditions present during the storage study. Ethylene concentrations ranged from 0.15 to 0.7 ppm. The highest observed ethylene concentration was observed for the 20°C apples at 50% relative humidity. Ethylene concentrations of 0.2 and 0.15 ppm were observed for the 4°C 89% relative humidity and 4°C 50% relative humidity, respectively. To put these values in perspective, far lower ethylene concentrations are commonly present during fruit ripening, ranging from 10 to 100 ppm as reported by Kupferman (2018), and a reported average value of 80 ppm for McIntosh apples just before peak ripening stage as observed by Burg and Burg) 196). Prior to ripening, the rate of ethylene production is generally low, usually
under 0.15 ppm (Kupferman 2018). It is also known that the accelerated production of ethylene occurs during the process of fruit ripening, though the effects of ethylene concentration on *L. monocytogenes* growth on apples during long term storage has not yet been studied. The observed ethylene concentrations make sense, considering that the apples used were already ripe. Thus, any possible effects of ethylene on *L. monocytogenes* during this experiment can be ruled out.

### 4.5 Conclusions

Low humidity leads to higher population reductions of *L. monocytogenes* than high humidity at the same temperature. Population reductions observed for untreated apples during the storage study at ~35% relative humidity discussed in chapter 3 were over 1 log CFU higher than those observed for untreated apples during the storage study at 53% and 89% relative humidity in this chapter. Findings for low humidity apples may not be significant use to the industry, given that apples are not stored at low humidity due to moisture loss and resultant quality decline.

Because long term storage under industry temperature and humidity conditions for up to 28 days cannot be relied upon to reduce *L. monocytogenes* populations, packers should take precautions to avoid contamination from occurring in the orchard through adherence with Good Agricultural Practices (GAPs) and in the packing house by following Good Handling Practices (GHP). Packers should particularly take care to minimize wounding on apples, considering that once apples are shipped, they can be exposed to environmental conditions that could lead to *L. monocytogenes* survival or growth. Taking into account this absence of effective hurdles during storage, alternative
methods for keeping *L. monocytogenes* at low levels should be considered, such as through the use of sanitizers in dump tanks and flumes. Therefore, in Chapter 5, the effect of sanitizers on *L. monocytogenes* populations on apples will be studied.
**Figure 4.1.** *Listeria monocytogenes* populations on intact McIntosh apples stored for up to 28 days at 4°C and 89 ± 1% RH, 20°C or 4°C and 50 ± 3% RH, and 20°C and 50 ± 3% RH. Mean ± SD, n=18.
Figure 4.2. *Listeria monocytogenes* populations on intact Fuji apples stored for up to 28 days 4°C and 89 ± 1% RH, 20°C or 4°C and 50 ± 3% RH, and 20°C and 50 ± 3% RH. Mean ± SD, n=18.
**Figure 4.3.** *Listeria monocytogenes* populations on wounded McIntosh apples stored for up to 28 days at 4°C and 89 ± 1% RH, 20°C or 4°C and 50 ± 3% RH, and 20°C and 50 ± 3% RH. Mean ± SD, n=18.
Figure 4.4. *Listeria monocytogenes* populations on wounded Fuji apples stored for up to 28 days at 4°C and 89 ± 1% RH, 20°C or 4°C and 50 ± 3% RH, and 20°C and 50 ± 3% RH. Mean ± SD, n=18.
Table 4.1. *Listeria monocytogenes* populations determined on MOX agar on intact and wounded McIntosh and Fuji apples stored for up to 28 days at 4°C and 89% RH. Values in parentheses at each time interval are population changes from apples measured before storage. Mean ± SD, n=18.

<table>
<thead>
<tr>
<th></th>
<th>McIntosh (intact)</th>
<th></th>
<th>Fuji (intact)</th>
<th></th>
<th>McIntosh (wounded)</th>
<th></th>
<th>Fuji (wounded)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Time (days)</strong></td>
<td><strong>T=0</strong></td>
<td><strong>T=7</strong></td>
<td><strong>T=14</strong></td>
<td><strong>T=21</strong></td>
<td><strong>T=28</strong></td>
<td><strong>T=0</strong></td>
<td><strong>T=7</strong></td>
<td><strong>T=14</strong></td>
</tr>
<tr>
<td></td>
<td>4.08 ± 0.11</td>
<td>3.75 ± 0.25</td>
<td>3.62 ± 0.08</td>
<td>3.85 ± 0.45</td>
<td>4.27 ± 0.14</td>
<td>4.09 ± 0.07</td>
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<td>(0.00)</td>
<td>(-0.33)</td>
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<td>(-0.23)</td>
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<td>(-0.18)</td>
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<tr>
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<td>4.38 ± 0.11</td>
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<td>3.95 ± 0.09</td>
<td>3.79 ± 0.14</td>
<td>4.47 ± 0.14</td>
<td>4.35 ± 0.10</td>
<td>3.93 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>(0.00)</td>
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<td>(-0.43)</td>
<td>(0.00)</td>
<td>(-0.12)</td>
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<td>(-0.55)</td>
</tr>
</tbody>
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**Table 4.2.** *Listeria monocytogenes* populations determined on MOX agar on intact and wounded McIntosh and Fuji apples stored for up to 28 days at 4°C and 53% RH. Values in parentheses at each time interval are population changes from apples measured before storage. Mean ± SD, n=18.

<table>
<thead>
<tr>
<th>Time (days)</th>
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<th>Fuji (intact)</th>
<th>McIntosh (wounded)</th>
<th>Fuji (wounded)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T=0</td>
<td>T=7</td>
<td>T=14</td>
<td>T=21</td>
</tr>
<tr>
<td></td>
<td>4.08 ± 0.11</td>
<td>3.55 ± 0.25</td>
<td>3.60 ± 0.06</td>
<td>3.57 ± 0.14</td>
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<tr>
<td></td>
<td>(-0.53)</td>
<td>(-0.48)</td>
<td>(-0.51)</td>
<td>(-0.51)</td>
</tr>
<tr>
<td></td>
<td>4.21 ± 0.11</td>
<td>3.68 ± 0.38</td>
<td>3.96 ± 0.06</td>
<td>3.84 ± 0.14</td>
</tr>
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<td>(-0.25)</td>
<td>(-0.37)</td>
<td>(-0.77)</td>
</tr>
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</table>

*Listeria monocytogenes* (log CFU/cm³) ± SD
Table 4.3. *Listeria monocytogenes* populations determined on MOX agar on intact and wounded McIntosh and Fuji apples stored for up to 28 days at 20°C and 50% RH. Values in parentheses at each time interval are population changes from apples measured before storage. Mean ± SD, n=18.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>McIntosh (intact)</th>
<th>Fuji (intact)</th>
<th>McIntosh (wounded)</th>
<th>Fuji (wounded)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T=0</td>
<td>T=7</td>
<td>T=14</td>
<td>T=21</td>
</tr>
<tr>
<td></td>
<td>4.30 ± 0.11</td>
<td>4.02 ± 0.07</td>
<td>3.74 ± 0.20</td>
<td>3.95 ± 0.05</td>
</tr>
<tr>
<td></td>
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<td>(-0.56)</td>
<td>(-0.35)</td>
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<tr>
<td></td>
<td>4.46 ± 0.11</td>
<td>4.17 ± 0.05</td>
<td>4.95 ± 0.22</td>
<td>4.22 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>(0.29)</td>
<td>(0.49)</td>
<td>(0.21)</td>
<td>(0.24)</td>
</tr>
</tbody>
</table>
**Table 4.4.** Total plate count (TPC) populations recovered on TSA agar on intact and wounded McIntosh and Fuji apples stored for up to 28 days at 4°C and 89% RH. Values in parentheses at each time interval are population changes from apples measured before storage. Mean ± SD, n=18.

<table>
<thead>
<tr>
<th></th>
<th>McIntosh (intact)</th>
<th>Fuji (intact)</th>
<th>McIntosh (wounded)</th>
<th>Fuji (wounded)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Listeria monocytogenes</strong> (log CFU/cm³) + SD</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td><strong>Time (days)</strong></td>
<td><strong>T=0</strong></td>
<td><strong>T=7</strong></td>
<td><strong>T=14</strong></td>
<td><strong>T=21</strong></td>
</tr>
<tr>
<td><strong>McIntosh (intact)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T=0</td>
<td>4.16 ± 0.15</td>
<td>3.74 ± 0.01 (-0.42)</td>
<td>3.60 ± 0.25 (-0.56)</td>
<td>3.78 ± 0.08 (-0.38)</td>
</tr>
<tr>
<td><strong>McIntosh (wounded)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T=0</td>
<td>4.16 ± 0.15</td>
<td>4.23 ± 0.17 (0.07)</td>
<td>3.82 ± 0.26 (-0.34)</td>
<td>4.15 ± 0.20 (-0.01)</td>
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</tbody>
</table>
Table 4.5. Total plate count (TPC) populations recovered on TSA agar on intact and wounded McIntosh and Fuji apples stored for up to 28 days at 4°C and 50% RH. Values in parentheses at each time interval are population changes from apples measured before storage. Mean ± SD, n=18.

<table>
<thead>
<tr>
<th>Time (days)</th>
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<th>Fuji (intact)</th>
<th>McIntosh (wounded)</th>
<th>Fuji (wounded)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
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</tr>
<tr>
<td>T=0</td>
<td>4.16 ± 0.15</td>
<td>4.31 ± 0.15</td>
<td>4.16 ± 0.15</td>
<td>4.30 ± 0.26</td>
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<td></td>
<td>(0.86)</td>
<td>(0.56)</td>
<td>(0.10)</td>
<td>(0.01)</td>
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<tr>
<td>T=7</td>
<td>3.30 ± 0.25</td>
<td>3.60 ± 0.14</td>
<td>4.06 ± 0.38</td>
<td>4.82 ± 0.56</td>
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<tr>
<td></td>
<td>(-0.66)</td>
<td>(-0.14)</td>
<td>(-0.14)</td>
<td>(0.51)</td>
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<tr>
<td>T=14</td>
<td>3.60 ± 0.46</td>
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<td>4.02 ± 0.06</td>
<td>4.61 ± 0.78</td>
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<td>(-0.56)</td>
<td>(-0.18)</td>
<td>(-0.14)</td>
<td>(0.30)</td>
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<tr>
<td>T=21</td>
<td>3.60 ± 0.03</td>
<td>3.60 ± 0.03</td>
<td>3.98 ± 0.14</td>
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<td>(-0.18)</td>
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<td>T=28</td>
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<td>3.60 ± 0.03</td>
<td>3.48 ± 0.08</td>
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<td>(-0.56)</td>
<td>(-0.56)</td>
<td>(-0.68)</td>
<td>(0.51)</td>
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*Listeria monocytogenes* (log CFU/cm$^3$) ± SD
Table 4.6. Total plate count (TPC) populations recovered on TSA agar on intact and wounded McIntosh and Fuji apples stored for up to 28 days at 20°C and 50% RH. Values in parentheses at each time interval are population changes from apples measured before storage. Mean ± SD, n=18.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>McIntosh (intact)</th>
<th>Fuji (intact)</th>
<th>McIntosh (wounded)</th>
<th>Fuji (wounded)</th>
</tr>
</thead>
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<tr>
<td></td>
<td>T=0</td>
<td>T=7</td>
<td>T=14</td>
<td>T=21</td>
</tr>
<tr>
<td>McIntosh</td>
<td>4.31 ± 0.15</td>
<td>4.16 ± 0.33</td>
<td>4.08 ± 0.07</td>
<td>3.93 ± 0.19</td>
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<tr>
<td></td>
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<td>(-0.23)</td>
<td>(-0.38)</td>
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<td>4.76 ± 0.60</td>
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<td>(0.45)</td>
<td>(1.11)</td>
<td>(0.57)</td>
<td>(0.04)</td>
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Table 4.7. Total plate count (TPC) populations recovered on TSA agar on intact and wounded McIntosh and Fuji apples stored for up to 28 days at 4°C and 89 ± 1% RH. Values in parentheses at each time interval are population changes from apples measured before storage. Mean ± SD, n=18.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>McIntosh (intact)</th>
<th>Fuji (intact)</th>
<th>McIntosh (wounded)</th>
<th>Fuji (wounded)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T=0</td>
<td>T=7</td>
<td>T=14</td>
<td>T=21</td>
</tr>
<tr>
<td></td>
<td>1.60 ± 0.12</td>
<td>1.00 ± 0.18</td>
<td>1.78 ± 0.07</td>
<td>1.39 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>(-0.60)</td>
<td>(0.18)</td>
<td>(-0.21)</td>
<td>(-0.60)</td>
</tr>
<tr>
<td></td>
<td>1.74 ± 0.37</td>
<td>1.00 ± 0.00</td>
<td>1.74 ± 0.37</td>
<td>1.37 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>(-0.74)</td>
<td>(0.00)</td>
<td>(0.00)</td>
<td>(-0.37)</td>
</tr>
</tbody>
</table>
Table 4.8. Total plate count (TPC) populations recovered on TSA agar on intact and wounded McIntosh and Fuji apples stored for up to 28 days at 4°C and 50 ± 3% RH. Values in parentheses at each time interval are population changes from apples measured before storage. Mean ± SD, n=18.

<table>
<thead>
<tr>
<th></th>
<th>Listeria monocytogenes (log CFU/cm³) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>McIntosh (intact)</td>
</tr>
<tr>
<td></td>
<td>Fuji (intact)</td>
</tr>
<tr>
<td>Time (days)</td>
<td>T=0</td>
</tr>
<tr>
<td>McIntosh (intact)</td>
<td>1.30 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>(-0.30)</td>
</tr>
<tr>
<td></td>
<td>McIntosh (wounded)</td>
</tr>
<tr>
<td></td>
<td>Fuji (wounded)</td>
</tr>
<tr>
<td>Time (days)</td>
<td>T=0</td>
</tr>
<tr>
<td>McIntosh (wounded)</td>
<td>1.30 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>(-0.30)</td>
</tr>
</tbody>
</table>
Table 4.9. Total plate count (TPC) populations recovered on TSA agar on intact and wounded McIntosh and Fuji apples stored for up to 28 days at 20°C and 50 ± 3% RH. Values in parentheses at each time interval are population changes from apples measured before storage. Mean ± SD, n=18.

<table>
<thead>
<tr>
<th></th>
<th>Listeria monocytogenes (log CFU/cm³) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>McIntosh (intact)</strong></td>
</tr>
<tr>
<td><strong>Time (days)</strong></td>
<td><strong>T=0</strong></td>
</tr>
<tr>
<td>McIntosh (intact)</td>
<td>1.60 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>(-0.20)</td>
</tr>
<tr>
<td>McIntosh (wounded)</td>
<td>1.48 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>(-0.18)</td>
</tr>
</tbody>
</table>
Table 4.10. Microbial populations determined on MOX, TAL, and TSA on intact and wounded McIntosh and Fuji apples stored for 21 and 28 days at 4°C and 89 ± 1% RH.

| Growth medium | McIntosh (intact) | | Fuji (intact) | |
|---------------|------------------|-----------------|-----------------|
|               | Time = 21 days   | Time = 28 days  | Time = 21 days  | Time = 28 days  |
| MOX           | 3.54 ± 0.16      | 3.40 ± 0.45     | 3.81 ± 0.04     | 3.85 ± 0.04     |
| TAL           | 3.62 ± 0.16      | 3.40 ± 0.45     | 3.83 ± 0.04     | 3.87 ± 0.04     |
| TSA           | 3.78 ± 0.08      | 3.85 ± 0.45     | 3.87 ± 0.02     | 3.90 ± 0.02     |

| McIntosh (wounded) | | Fuji (wounded) | |
|-------------------|-----------------|-----------------|
|                   | Time = 21 days  | Time = 28 days  | Time = 21 days  | Time = 28 days  |
| MOX               | 3.74 ± 0.21     | 3.70 ± 0.09     | 3.81 ± 0.11     | 3.88 ± 0.39     |
| TAL               | 3.95 ± 0.21     | 3.79 ± 0.18     | 3.92 ± 0.11     | 4.26 ± 0.38     |
| TSA               | 4.15 ± 0.20     | 3.88 ± 0.09     | 4.02 ± 0.10     | 4.63 ± 0.38     |

% Injury: 8.4% for McIntosh and 28.6% for Fuji.
Table 4.11. Microbial populations determined on MOX, TAL, and TSA on intact and wounded McIntosh and Fuji apples stored for 21 and 28 days at 4°C and 50 ± 3% RH. Mean ± SD, n=18.

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>McIntosh (intact)</th>
<th></th>
<th>Fuji (intact)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time = 21 days</td>
<td>Time = 28 days</td>
<td></td>
<td>Time = 21 days</td>
</tr>
<tr>
<td>MOX</td>
<td>3.54 ± 0.06</td>
<td>3.54 ± 0.06</td>
<td>% Injury</td>
<td>3.81 ± 0.00</td>
</tr>
<tr>
<td>TAL</td>
<td>3.57 ± 0.06</td>
<td>3.57 ± 0.06</td>
<td>6.7%</td>
<td>3.81 ± 0.15</td>
</tr>
<tr>
<td>TSA</td>
<td>3.60 ± 0.03</td>
<td>3.60 ± 0.03</td>
<td>4.10 ± 0.19</td>
<td>4.28 ± 0.32</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>McIntosh (wounded)</th>
<th></th>
<th>Fuji (wounded)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Time = 21 days</td>
<td>Time = 28 days</td>
<td>Time = 21 days</td>
<td>Time = 28 days</td>
</tr>
<tr>
<td>MOX</td>
<td>3.70 ± 0.14</td>
<td>3.40 ± 0.08</td>
<td>3.74 ± 0.08</td>
</tr>
<tr>
<td>TAL</td>
<td>3.84 ± 0.14</td>
<td>3.44 ± 0.08</td>
<td>3.78 ± 0.08</td>
</tr>
<tr>
<td>TSA</td>
<td>3.98 ± 0.14</td>
<td>3.48 ± 0.08</td>
<td>3.81 ± 0.06</td>
</tr>
</tbody>
</table>
Table 4.12. Microbial populations determined on MOX, TAL, and TSA on intact and wounded McIntosh and Fuji apples stored for 21 and 28 days at 20°C and 50 ± 3% RH. Mean ± SD, n=18.

<table>
<thead>
<tr>
<th>Growth medium</th>
<th>McIntosh (intact)</th>
<th>Fuji (intact)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time = 21 days</td>
<td>Time = 28 days</td>
</tr>
<tr>
<td>MOX</td>
<td>3.54 ± 0.20</td>
<td>3.90 ± 0.10</td>
</tr>
<tr>
<td>TAL</td>
<td>3.74 ± 0.20</td>
<td>3.95 ± 0.10</td>
</tr>
<tr>
<td>TSA</td>
<td>3.93 ± 0.19</td>
<td>4.00 ± 0.05</td>
</tr>
</tbody>
</table>

% Injury 23.9%

<table>
<thead>
<tr>
<th>McIntosh (wounded)</th>
<th>Fuji (wounded)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time = 21 days</td>
<td>Time = 28 days</td>
</tr>
<tr>
<td>MOX</td>
<td>4.45 ± 0.22</td>
</tr>
<tr>
<td>TAL</td>
<td>4.67 ± 0.22</td>
</tr>
<tr>
<td>TSA</td>
<td>4.88 ± 0.21</td>
</tr>
</tbody>
</table>

% Injury 33.7%
Table 4.13. Mean gas compositions measured at 21 and 28 days storage. Mean ± SD, n=3.

<table>
<thead>
<tr>
<th>Storage conditions</th>
<th>Oxygen (%)</th>
<th>Carbon Dioxide (PPM)</th>
<th>Ethylene (PPM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4°C, 89 ± 1% RH</td>
<td>20 ± 1</td>
<td>750 ± 13</td>
<td>0.25 ± 0.05</td>
</tr>
<tr>
<td>4°C, 50 ± 3% RH</td>
<td>21 ± 1</td>
<td>740 ± 23</td>
<td>0.15 ± 0.05</td>
</tr>
<tr>
<td>20°C, 50 ± 3% RH</td>
<td>20 ± 2</td>
<td>800 ± 37</td>
<td>0.7 ± 0.1</td>
</tr>
</tbody>
</table>

Table 4.14. pH and total soluble solids (Brix) of apples. Mean ± SD, n=3.

<table>
<thead>
<tr>
<th>Apple Variety</th>
<th>McIntosh</th>
<th>Fuji</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>3.29 ± 0.04</td>
<td>3.6 ± 0.24</td>
</tr>
<tr>
<td>Total soluble solids (TSS) %</td>
<td>11.5 ± 0.3</td>
<td>13.3 ± 0.3</td>
</tr>
</tbody>
</table>
Table 4.15. Summary of results from storage experiments 1 and 2.

<table>
<thead>
<tr>
<th>Storage Experiment</th>
<th>Temperature (°C)</th>
<th>Relative Humidity*</th>
<th>*Listeria monocytogenes Log Reduction (wounded)</th>
<th>*Listeria monocytogenes Log Reduction (intact)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
<td>~35</td>
<td>1.72</td>
<td>1.67</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>53</td>
<td>0.59</td>
<td>0.47</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>89</td>
<td>0.40</td>
<td>0.32</td>
</tr>
<tr>
<td>2</td>
<td>20</td>
<td>50</td>
<td>0.13</td>
<td>0.68</td>
</tr>
</tbody>
</table>

* RH measured after experiment 1 completed
CHAPTER 5:

EFFECT OF SANITIZERS ON *LISTERIA MONOCYTOGENES* INOCULATED 
APPLES AND ON CROSS-CONTAMINATION OF NON-INOCULATED 
APPLES IN A SIMULATED DUMP TANK

5.1 Abstract

A study was performed to determine the effectiveness of sanitizers on reducing populations of *L. monocytogenes* on inoculated apples and their ability to prevent cross-contamination to non-inoculated apples in a simulated dump tank. Initial *L. monocytogenes* population levels 6.7 log CFU/mL for the chlorine-based sanitizer experiments and 6.47 log CFU/mL for the peroxycetic acid/hydrogen peroxide sanitizer experiments. Apples immersed in sodium hypochlorite (100, 150, or 200 ppm OCl\(^-\), adjusted or not adjusted with citric acid to pH 6.8), sodium chlorite, and peroxycetic acid (PAA) / hydrogen peroxide formulations (60, 80, or 100 ppm PPA) for 5 minutes were studied. There were no significant (p=0.05) reductions of *L. monocytogenes* on inoculated apples treated with sodium hypochlorite at the un-adjusted pH of 8.7-9.0. However, when solutions were adjusted to pH 6.8, population reductions were significantly higher (p=0.05) compared to the control as Oxidation Reduction Potential (ORP) values approximately doubled. Unadjusted and adjusted pH sodium hypochlorite solutions decreased *L. monocytogenes* cross contamination to non-inoculated apples, except for 100 ppm at pH 8.7. Similar to low pH adjusted sodium hypochlorite solutions, peroxycetic acid/hydrogen peroxide sanitizers (SaniDate products), with the exception
of SaniDate FD at 60 ppm PAA, were significantly more effective at limiting cross contamination compared to the control. In addition to these findings, SaniDate products were more effective at reducing *L. monocytogenes* populations than unadjusted pH sodium hypochlorite products. It should be noted that for all sanitizers tested, complete prevention of cross contamination was not possible and concentration did significantly affect population reductions. Therefore, the addition of sanitizers during dump tanks or flumes is preferred over using untreated water for the control of *L. monocytogenes*.

### 5.2 Introduction

The incidence of outbreaks and recalls has led to a need to conduct research on sanitizer effectiveness in solutions used to wash or transport fresh produce. The most commonly used sanitizers are chlorine-based, though peroxycetic acid and hydrogen peroxide sanitizers are gaining in popularity (Rodgers et al. 2004). A maximum of 2000 ppm (0.2%) sodium hypochlorite is allowed in wash water, according to the US Code of Federal Regulations (Title 21 CFR Section 178.1010), although water containing 50 to 200 ppm hypochlorite is widely used in fruit and vegetable wash waters on a commercial scale (Beuchat et al. 1998). A previous study evaluating the effectiveness of peroxycetic acid sanitizers against *L. monocytogenes* populations on fresh apples, lettuce, strawberries, and cantaloupe demonstrated that a concentration of 80 ppm peroxycetic acid resulted in 5-log reductions or greater on the initial produce inoculum level of $10^6$ CFU/mL (Rodgers et al. 2004). The use of 80 ppm peroxycetic acid is in line with the EPA limit of 100 ppm of peroxycetic acid for use on fresh produce (Title 40 CFR Section 180.1196). The same study reported similar *L. monocytogenes* reductions using chlorine dioxide at 3 and 5 ppm (Rodgers et al. 2004), which is within
the EPA limit of 5 ppm for chlorine dioxide used in produce wash water (Title 21 CFR Section 173.300). A shortcoming of these studies is that the authors did not evaluate the effect of hypochlorite pH and ORP on *L. monocytogenes* populations on fresh produce, apples in particular. The upcoming study was done to determine the effects of sanitizer application on pathogen reduction on inoculated apples, as well as the effects of sanitizer application on cross-contamination between inoculated and non-inoculated apples. Lowering the pH to raise ORP of sodium hypochlorite increases pathogen reduction due to the fact that sodium hypochlorite activity is greater when hypochlorite is protonated. Protonated hypochlorite is better able to disrupt bacterial cell membranes than non-protonated hypochlorite, leading us to predict that low pH, high ORP hypochlorite solutions will produce higher population reductions than high pH, low ORP hypochlorite solutions.

### 5.3 Materials and Methods

#### 5.3.1 Strain Selection and Preparation of Working Cultures

A 5-strain cocktail of a *L. monocytogenes* overnight culture was prepared from isolates taken from several fresh cut apple facilities where *L. monocytogenes* was detected. The cocktail consisted of strains of serotype 4b, serotype 1/2a, and serotype 1/2b all of stock source. Three strains were isolated from a tree fruit packing non-food-contact environment, 1 from a food contact surface at a facility involved in a recall, and the other a clinical sample. The working culture was prepared in tryptic soy broth yeast extract (TSBYE) as described by Jones and D’Orazio (2013), where for each strain, a single colony was inoculated into 50 mL TSBYE and incubated for 48 hours at 35.5°C.
The optical density for each strain was adjusted to 0.5 to produce a concentration of $10^9$ CFU/mL. Ten mL of each of the working cultures for the different five strains were combined into a sterile beaker to form the working culture.

5.3.2. Preparation of Inoculum

Thirty 1-mL centrifuge tubes were each filled with 1 mL of the combined working culture. Each tube was centrifuged for 3 minutes at 10000 x g, after which, the supernatant was decanted and replaced with 1 mL of 0.85% sodium chloride (NaCl) and then spun down again. The wash process was repeated once. The washed mixtures were then vortexed to evenly distribute *L. monocytogenes* cells in the tube and then all of the individual tubes were combined in a beaker. The concentration of *L. monocytogenes* in the inoculum was $10^9$ CFU/mL. Actual initial inoculum levels were determined by plating before each experiment was conducted.

5.3.3. Inoculation and Treatment of Apples

Unwaxed apples (var. McIntosh) were obtained from a local apple packing facility. Six 1-mL centrifuge tubes were each filled with 1 mL of the overnight culture. The tubes were centrifuged for 3 minutes at 10000 x g to spin the *L. monocytogenes* cells to the bottom of the tube. For each tube, the supernatant was decanted and replaced with 1 mL of 0.85% sodium chloride (NaCl) and spun down again. The wash process was repeated once.

The mixture was then vortexed to evenly distribute *L. monocytogenes* in the tube and the contents of each tube were then added to 3 L of sterile 0.85% NaCl. Considering that the starting concentration of the *L. monocytogenes* overnight culture
was of $10^9$ CFU/mL, the addition of the contents of each tube to 3 L of 0.85% NaCl created a new *L. monocytogenes* concentration of $2 \times 10^6$ CFU/mL, which was later confirmed by plating on MOX agar. For each sanitizer treatment, three replicate apples were dip-inoculated in the inoculation solution for 5 minutes and placed on a paper tray to air dry for 1 hour at room temperature. Ten L plastic containers were filled with 2 L of each of the various sanitizers or water as a control. Three un-inoculated apples were added along with three inoculated apples and held for 5 minutes, given that this the amount of time that apples would exposed under normal commercial packing conditions. Gentle stirring was done to ensure that each apple was equally submerged in the solution. This entire process was repeated twice.

### 5.3.4. Preparation of Sanitizer Solutions

Peroxyacetic / hydrogen peroxide formulations (SaniDate FD™, SaniDate 5.0, SaniDate 15.0, BioSafe Sysems, East Hartford, CN) were prepared according to label directions by diluting with distilled water to achieve PA levels of 60, 80, and 100 PPM. Sodium hypochlorite (Clorox, Oakland, CA) (pH 8.7 or 6.8) was diluted to 100 PPM free chlorine. Distilled water was used as a control. Chlorine dioxide (Keeper, Bio-Cide Int. Laboratory, Norman, OK) was prepared by mixing 5.6 mL sodium chlorite with 0.6 g citric acid and then adding to 1.89 L of distilled water to achieve a concentration of 5 PPM. For each treatment, 2.0 L of sanitizer was added to 10-L plastic containers. pH was measured used pHydrion® pH paper. Sanitizer Oxidation Reduction Potential (ORP) was measured before and apple immersion using an ORP meter (Orion Redox, ThermoFisher Scientific).
5.3.5. Experimental design and statistical analysis

Uninoculated apples were compared to an initial *L. monocytogenes* population of zero. Values were analyzed using 1-way ANOVA. Visible recovered colonies were counted and counts were multiplied by their respective dilution factor to get population values in terms of log CFU/mL.

5.3.6. *L. monocytogenes* enumeration

After treatment, apples were individually placed into large stomacher bags (Whirlpack ®) filled with 100 mL of D/E neutralizing broth and massaged for 5 minutes. After massaging, apples were taken out and serially diluted in sterile 0.85% NaCl (10⁻¹, 10⁻², and 10⁻³) for plating (100 µL) on modified Oxford (MOX) agar for incubation at 35.5°C for 20 hours. Population counts were multiplied by their respective dilution factor to get population values in terms of log CFU/mL. The entire process was repeated once.

5.4. Results and Discussion

5.4.1. Chlorine Sanitizers

Figure 5.1, Figure 5.2, and Table 5.2 show the effects of sodium hypochlorite (100, 150, and 200 ppm) and chlorine dioxide (5 ppm) on *Listeria monocytogenes* populations on both inoculated and non-inoculated apples submerged in the simulated dunk tank. Populations were tracked on inoculated apples to evaluate the reducing power of the sanitizers and populations were tracked on non-inoculated apples to determine the extent of cross-contamination.
It was initially hypothesized that sanitizers would prevent cross contamination from occurring, but not significantly reduce *Listeria monocytogenes* populations on inoculated apples. However, with the exception of high pH, low ORP sodium hypochlorite sanitizers, significant, population reductions occurred on inoculated apples (p=0.05). Sanitizer treatments and the control (deionized water) failed to prevent cross-contamination from taking place, but chlorine sanitizers used were significantly more effective at limiting cross contamination than the control. Although significant population reductions were obtained for all chlorine sanitizers used, all sanitizers used fell short of the acceptable industry standard of a 5-log reduction, with the largest pathogen reductions being 4.6 log CFU/mL for low pH, high ORP sodium hypochlorite at 200 ppm. This reduction was greater than the 0.95 log reduction reported by Beuchat et al. (1998) at a 200 ppm concentration (Beuchat et al. 1998), although the authors did not specify the pH and ORP of the sanitizer. An encouraging sign was that with the exception of high pH, low ORP hypochlorite at 100 ppm, all chlorine sanitizers used were significantly more effective at limiting cross contamination than the control. Despite achieving significant population reductions on inoculated apples (p=0.05), hypochlorite sanitizer concentration had no significant effect on the population reductions achieved (p=0.05).

Sodium hypochlorite solutions at low pH (6.8) and high ORP (730-780 mV) were the most effective chlorine sanitizers used (p=0.05), followed by chlorine dioxide at 5 ppm, with sodium hypochlorite sanitizers at high pH (8.7-9.0) and low ORP (420-450 mV) being the least effective (p=0.05). Figure 5.1, Figure 5.2, and Table 5.2 show that population reductions observed by sodium hypochlorite solutions at high pH (8.7-9.0)
and low ORP (420-450 mV) were significantly lower (p<0.05) than the 3.75 log reductions observed by chlorine dioxide at 5 ppm. The chlorine dioxide population reductions were greater than the reported reductions of 3 log CFU by Wisniewsky et al. (2000), but lower than the reported findings of Rodgers et al. (2004) where over a 5-log reduction of *Listeria monocytogenes* populations was observed. Population reductions observed by sodium hypochlorite solutions at lower pH (6.8) and high ORP (730-780 mV) were significantly higher (p=0.05) than those observed by sodium hypochlorite solutions at high pH (8.7-9.0) and low ORP (420-450 mV), illustrating that lowering the pH to drive up ORP drastically improves *Listeria monocytogenes* reducing power.
5.4.2. Peroxyacetic Acid/Hydrogen Peroxide Sanitizers

Figure 5.3 and Table 5.3 show the effects of peroxyacetic acid/hydrogen peroxide sanitizers on *Listeria monocytogenes* populations on both inoculated and non-inoculated apples. SaniDate 5.0, SaniDate 15.0, and SaniDate FD at all concentrations tested (60, 80, and 100 ppm) generated significant population reductions on inoculated apples (p=0.05). Like chlorine sanitizers, peroxyacetic acid/hydrogen peroxide sanitizers were unable to completely prevent cross-contamination from occurring, given that significant population increases (p=0.05) on uninoculated apples occurred. Although significant population reductions were obtained for all SaniDate products used, each of the sanitizers fell short of the acceptable industry standard of a 5-log reduction, as the largest pathogen reduction was just under 4 log CFU/mL. Also like chlorine sanitizers, with the exception of SaniDate FD at 60 PPM PAA all SaniDate products used were significantly more effective at limiting cross contamination than the control.

As seen in Tables 5.1 and 5.3, there was no significant difference between SaniDate products in terms of the population reduction achieved (p=0.05). Also, no significant difference in population reductions between different sanitizers of the same concentration was observed (p=0.05) on inoculated apples, with the one exception being SaniDate FD and SaniDate 15 at 60 ppm. The reductions obtained from these sanitizers contradict the findings reported by Rodgers et al. (2004) where they observed over a 5-log reduction of *L. monocytogenes* populations on whole apples after treatment for 5 minutes with peroxyacetic acid at 80 ppm. Interestingly, population reductions observed for SaniDate products were comparable to population reductions observed by Wisniewsky et al, where they observed 3 log CFU for peroxyacetic acid at 80 ppm. As
seen in Tables 5.2 and 5.3, SaniDate products had population reductions significantly higher (p=0.05) than those observed by sodium hypochlorite solutions at high pH (8.7-9.0) and low ORP (420-450 mV), but significantly lower (p=0.05) than sodium hypochlorite solutions at lower pH (6.8) and high ORP (730-780 mV).

In summary, observations from this study appear to contradict those from previous studies that show that sodium hypochlorite solutions (100-200 ppm chlorine) are not as effective as peroxyacetic acid solutions at reducing *Listeria monocytogenes* populations, though those other studies did not specify the pH and ORP of chlorine sanitizers that were used (Beuchat et al 1998, Rodgers et al 2004).

### 5.5 Conclusions

Considering that none of the sanitizers used were able to prevent cross-contamination from occurring, produce packers need to be cognizant of their handling of apples before bringing them to dump tanks, in order to limit the amount of contamination that takes place before sanitizer application. When using hypochlorite sanitizer in a dump tank filled with apples, or similar type of produce, packers should be sure to adjust the pH of hypochlorite solutions in order to maximize ORP and therefore minimize pathogen populations. Due to comparable results observed for chlorine dioxide and peroxyacetic acid sanitizers, these sanitizers should produce similar results. In fact, one might prefer to use these compounds over hypochlorite solutions since a pH adjustment is not needed. Because there was no significant difference in effectiveness among SaniDate products, any one of the aforementioned SaniDate products will do.
In this study, the effect of sanitizer treatments was limited to just intact apple surfaces treated for 5 minutes, the approximate time apples would be exposed under normal commercial packing conditions. Given that it is known that sanitizing treatments fail to completely inactivate microbes in crevices and creases of produce due to lack of solution penetration, this may not be enough time for sanitizers to reach cells deep inside crevices and creases of the apple. Also microorganisms can become enmeshed in cuticle regions of produce, making removal difficult, thus reinforcing the need to avoid prior contamination before treatment application. It is recommended that apple growers engage in Good Agricultural Practices in order to prevent contamination, thus minimizing *Listeria monocytogenes* risks. Also, one should look to increase apple immersion time in dump tanks, looking to maximize pathogen and cross-contamination reductions.
Table 5.1. Sanitizers used in this study.

<table>
<thead>
<tr>
<th>Sanitizer</th>
<th>Sanitizer conc. (ppm)*</th>
<th>Label formulation</th>
<th>Ratio PAA/H₂O₂</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAA/H₂O₂ (5:23 ratio)</td>
<td>2048</td>
<td>5.3% PAA, 23% H₂O₂</td>
<td>0.230</td>
</tr>
<tr>
<td>PAA/H₂O₂ (15:10 ratio)</td>
<td>683</td>
<td>15% PAA, 10% H₂O₂</td>
<td>1.50</td>
</tr>
<tr>
<td>PAA/H₂O₂ (15:5 ratio)</td>
<td>683</td>
<td>15% PAA, 5% H₂O₂</td>
<td>3.00</td>
</tr>
<tr>
<td>Chlorine Dioxide</td>
<td>5</td>
<td>6% NaClO₂</td>
<td>N/A</td>
</tr>
<tr>
<td>Sodium Hypochlorite</td>
<td>100</td>
<td>8.3% NaClO₃</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*All SaniDate™ sanitizer concentrations were adjusted to their respective equivalent PAA levels.
Table 5.2. Effect of chlorine sanitizers on *Listeria monocytogenes* populations (log reductions in parentheses).

<table>
<thead>
<tr>
<th>Chlorine Product</th>
<th>Chlorine ppm</th>
<th>pH</th>
<th>Initial ORP (mV)</th>
<th>Final ORP (mV)</th>
<th>Inoculated Apples (log CFU/mL)</th>
<th>Non-inoculated Apples (log CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>7.0</td>
<td>250 ± 0</td>
<td>250 ± 0</td>
<td>*5.01 ± 0.02 (1.69)</td>
<td>**2.78 ± 0.11</td>
</tr>
<tr>
<td>ClO₂</td>
<td>5</td>
<td>5.5</td>
<td>890 ± 10</td>
<td>910 ± 10</td>
<td>**2.95 ± 0.04 (3.75)</td>
<td>***2.00 ± 0.18</td>
</tr>
<tr>
<td>HOCl</td>
<td>100</td>
<td>6.8</td>
<td>830 ± 10</td>
<td>850 ± 10</td>
<td>***2.24 ± 0.14 (4.46)</td>
<td>***1.48 ± 0.06</td>
</tr>
<tr>
<td>HOCl</td>
<td>100</td>
<td>8.7</td>
<td>410 ± 10</td>
<td>430 ± 10</td>
<td>*4.55 ± 0.38 (2.15)</td>
<td>**2.33 ± 0.21</td>
</tr>
<tr>
<td>HOCl</td>
<td>150</td>
<td>6.8</td>
<td>850 ± 10</td>
<td>870 ± 10</td>
<td>***2.15 ± 0.11 (4.55)</td>
<td>***1.56 ± 0.06</td>
</tr>
<tr>
<td>HOCl</td>
<td>150</td>
<td>8.9</td>
<td>430 ± 10</td>
<td>450 ± 10</td>
<td>*4.46 ± 0.32 (2.24)</td>
<td>***1.86 ± 0.29</td>
</tr>
<tr>
<td>HOCl</td>
<td>200</td>
<td>6.8</td>
<td>850 ± 10</td>
<td>890 ± 10</td>
<td>***2.10 ± 0.12 (4.60)</td>
<td>***1.56 ± 0.18</td>
</tr>
<tr>
<td>HOCl</td>
<td>200</td>
<td>9.0</td>
<td>450 ± 10</td>
<td>450 ± 10</td>
<td>*4.89 ± 0.03 (1.81)</td>
<td>***1.71 ± 0.07</td>
</tr>
</tbody>
</table>

Initial inoculated apples *Listeria monocytogenes* populations were log 6.7 ± 0.23 log CFU/ml. Treatment values in the same column with single asterisks are significantly different than values with double asterisks, which are significantly different than values with triple asterisks (p < 0.05).
Table 5.3. Effect of PAA/H₂O₂ sanitizers on *L. monocytogenes* populations (log reductions in parentheses)

<table>
<thead>
<tr>
<th>Sanitizer Used</th>
<th>PA ppm</th>
<th>pH</th>
<th>Initial ORP (mV)</th>
<th>Final ORP (mV)</th>
<th>Inoculated Apples (CFU/mL)</th>
<th>Non-inoculated Apples (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>7.0</td>
<td>250 ± 0</td>
<td>250 ± 0</td>
<td>*5.01 ± 0.02 (1.46)</td>
<td>**2.78 ± 0.11</td>
</tr>
<tr>
<td>SaniDate 15.0</td>
<td>60</td>
<td>5.5</td>
<td>760 ± 10</td>
<td>790 ± 10</td>
<td>**3.28 ± 0.14 (3.19)</td>
<td>**2.30 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>5.6</td>
<td>760 ± 10</td>
<td>790 ± 10</td>
<td>**2.75 ± 0.24 (3.72)</td>
<td>**1.36 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>5.8</td>
<td>750 ± 10</td>
<td>780 ± 10</td>
<td>**3.05 ± 0.24 (3.42)</td>
<td>**1.64 ± 0.19</td>
</tr>
<tr>
<td>SaniDate FD</td>
<td>60</td>
<td>5.9</td>
<td>750 ± 10</td>
<td>770 ± 10</td>
<td>**2.82 ± 0.27 (3.65)</td>
<td>**1.77 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>6.0</td>
<td>750 ± 10</td>
<td>780 ± 10</td>
<td>**2.80 ± 0.16 (3.67)</td>
<td>**1.76 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>6.1</td>
<td>760 ± 10</td>
<td>780 ± 10</td>
<td>**2.78 ± 0.15 (3.69)</td>
<td>**1.83 ± 0.35</td>
</tr>
<tr>
<td>SaniDate 5.0</td>
<td>60</td>
<td>6.2</td>
<td>760 ± 10</td>
<td>750 ± 10</td>
<td>**3.20 ± 0.20 (3.27)</td>
<td>**1.43 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>6.3</td>
<td>750 ± 10</td>
<td>740 ± 10</td>
<td>**2.64 ± 0.21 (3.83)</td>
<td>**1.78 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>6.5</td>
<td>730 ± 10</td>
<td>720 ± 10</td>
<td>**2.93 ± 0.17 (3.54)</td>
<td>**1.52 ± 0.38</td>
</tr>
</tbody>
</table>

Initial population on inoculated un-treated apple was 6.47 ± 0.23 log CFU/ml. Treatment values in the same column with single asterisks are significantly different than values with double asterisks, which are significantly different than values with triple asterisks (p < 0.05).
**Figure 5.1.** Effect of unadjusted pH chlorine sanitizers on apple *L. monocytogenes* populations. pH = 8.7. ORP = 420-450 mV.

Total chlorine ppm listed in parentheses next to chlorine sanitizer. Treatment values for inoculated apples marked with single asterisk (*) are significantly different at (p < 0.05) from the control values of inoculated apples. Treatment values for non-inoculated apples marked with a double asterisk (**) signify a significantly lower population increase (p=0.05) from zero compared to the population increase of the control.
**Figure 5.2.** Effect of adjusted pH chlorine sanitizers on apple *L. monocytogenes* populations. pH = 6.8. ORP 730-780 mV.

Total chlorine ppm listed in parentheses next to chlorine sanitizer. Treatment values for inoculated apples marked with single asterisk (*) are significantly different at (p < 0.05) from the control values of inoculated apples. Treatment values for non-inoculated apples marked with a double asterisk (**) signify a significantly lower population increase (p=0.05) from zero compared to the population increase of the control.
Figure 5.3. Effect of PAA/H$_2$O$_2$ sanitizers on apple *L. monocytogenes* populations. PAA ppm listed in parentheses next to PAA/H$_2$O$_2$ ratio of sanitizer.

Treatment values for inoculated apples marked with single asterisk (*) are significantly different at (p < 0.05) from the control values of inoculated apples. Treatment values for non-inoculated apples marked with a double asterisk (**) signify a significantly lower population increase (p=0.05) from zero compared to the population increase of the control.
CHAPTER 6:

CONCLUSIONS AND FUTURE DIRECTIONS

6.1 Conclusions

The first objective of the project was to determine whether carnauba wax and/or shellac coatings affect *L. monocytogenes* growth. A plate overlay assay was performed placing dried carnauba wax and shellac disks over a lawn of *L. monocytogenes*, looking for clearing zones of inhibition to shows anti-*Listeria* effects. No clearing zones were found, signifying that such coatings do not possess anti-*Listeria* ability.

The second objective of the project was to determine the effect of long term storage on *L. monocytogenes* populations on both wounded and intact fresh apples in the presence/absence of fruit coatings, fruit coating-antimicrobial emulsions, and aqueous antimicrobial solutions at specified temperatures and relative humidities. Significant differences in population reductions (p=0.05) were observed with apples treated with lauric arginate in carnauba and lauric arginate in shellac, as compared to the control for intact apples, but not for wounded apples. Results showed that compared to the control, there was no significant differences in population reduction between coating types, aligning with expectations based on plate overlay assay results. No significant population changes were observed between the temperatures and/or relative humidities studied after 28 days storage. Despite differences in physiochemical varieties, no difference in population changes was observed between apple varieties. It was also found that lower humidity leads to higher population reductions than higher...
humidity and that high temperature leads to lower population reductions than low temperature.

*Listeria monocytogenes* did not grow on apples during long term storage, regardless of whether treatments were applied. This is an important finding for the industry, indicating that long term storage is sufficient for keeping low *L. monocytogenes* population levels from increasing presumably because the pathogen does not utilize complex compounds in the coatings as a nutrient source that would support growth. Nevertheless, stable or low population reductions observed suggest that the industry cannot rely on long term storage to reduce high *L. monocytogenes* population levels to safe levels.

The third objective of the study was to determine the effect of commercial sanitizers on *L. monocytogenes* populations on fresh apples at modified pH and ORP conditions, as well as whether such sanitizers can prevent cross contamination of *L. monocytogenes* on fresh apples. In this study, it was found that sanitizers reduced *L. monocytogenes* populations on inoculated apples, with the exception of high pH, low ORP sodium hypochlorite solutions. The sanitizers were only partially effective at reducing cross contamination between apples, with the exception of high pH, low ORP sodium hypochlorite at 100 ppm, and SaniDate FD at 60 ppm PAA.

Because there was incomplete reduction and always some level of cross contamination observed in the simulated dump tank experiments, apple packers should take measures to prevent contamination such as obtaining assurances that growers are following Good Agricultural Practices in the orchard. In addition, packers should make
sure that they are complying with Good Manufacturing Practices within the facility in order to minimize the potential for *Listeria monocytogenes* to survive and grow.

### 6.2 Future Directions

For future research, one could study other antimicrobials to incorporate into coatings, provided that such antimicrobials have adequate solubility in the carnauba wax and shellac coatings. The reason for this is that the population reductions during storage obtained for lauric arginate incorporated into carnauba wax and shellac fell far below acceptable industry reductions of 5 log CFU. Given this information, one should seek more potent antimicrobials capable of wiping out high population levels.

Also, given that only 5 minutes treatment times were studied for the sanitizer study, future research should study longer dump tank treatment times in order to optimize pathogen reduction. The following recommendations should be followed: In the orchard, one should limit contamination in the field by keeping animals and manure away from apples and other produce. In controlled atmosphere storage, the effects of ozone and ClO₂ gas treatment could be studied to reduce contamination levels prior to packing. Also, within packing houses, methods for controlling moisture and improving drainage onto floors should be studied, as these sites have been shown to be areas of high *Listeria monocytogenes* contamination. Recommendations should be made on effective cleaning and sanitizing equipment and the design of equipment should improved to prevent against harborage sites and buildup of *Listeria monocytogenes*. Retailers should handle apples with care to prevent bruising and wounding and also store apples in bags, to prevent contamination and bacterial growth given results that
showed increased pathogen growth occurs with wounded apples under certain conditions. Finally, consumers should handle apples carefully and keep them refrigerated to minimize bacterial growth.
Appendix

Media Used and Formulations

Tryptase Soy Agar (TSA)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>17</td>
</tr>
<tr>
<td>Soytone</td>
<td>3</td>
</tr>
<tr>
<td>Dextrose</td>
<td>2.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>2.5</td>
</tr>
<tr>
<td>Agar</td>
<td>15</td>
</tr>
</tbody>
</table>

Ingredients were mixed with 1 L of deionized water and autoclaved for 33 minutes at 121°C.
Trypticase Soy Broth (TSB)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>17</td>
</tr>
<tr>
<td>Soytone</td>
<td>3</td>
</tr>
<tr>
<td>Dextrose</td>
<td>2.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>2.5</td>
</tr>
</tbody>
</table>

Ingredients were mixed with 1 L of deionized water and autoclaved for 33 minutes at 121°C.
Trypticase Soy Broth w/Yeast Extract (TSBYE)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptone</td>
<td>17</td>
</tr>
<tr>
<td>Soytone</td>
<td>3</td>
</tr>
<tr>
<td>Dextrose</td>
<td>2.5</td>
</tr>
<tr>
<td>NaCl</td>
<td>5</td>
</tr>
<tr>
<td>K$_2$HPO$_4$</td>
<td>2.5</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>5</td>
</tr>
</tbody>
</table>

Ingredients were mixed with 1 L of deionized water and autoclaved for 33 minutes at 121°C.
Modified Oxford (MOX)

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (g/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Columbia Agar Base</td>
<td>38.1</td>
</tr>
<tr>
<td>Lithium Chloride</td>
<td>15</td>
</tr>
<tr>
<td>Esculin</td>
<td>1</td>
</tr>
<tr>
<td>Ferric Ammonium Citrate</td>
<td>0.5</td>
</tr>
<tr>
<td>Colistin</td>
<td>0.01</td>
</tr>
<tr>
<td>Moxalactam</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Ingredients were mixed with 1 L of deionized water and autoclaved for 33 minutes at 121°C.
References


CDC. 2012. Multistate outbreak of listeriosis linked to whole cantaloupes from Jensen


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FDA. 2015a. Del Monte fresh produce N.A. Inc., recalls limited quantity of fresh apples due to possible health risk. U.S. Food and Drug Administration. October 14,


Pao, S. and, C. L. Davis. 1999. Enhancing microbiological safety of fresh orange juice by fruit immersion in hot water and chemical sanitizers. *Journal of Food*
Protection. 62(7):756–760.


monocytogenes in solution and on apples, lettuce, strawberries, and cantaloupe.


Technical Evaluation Report. Available at:

Vandekinderen, I., F. Devlieghere, B. De Meulenaer, P. Ragaert, and J. Van Camp.


