THE EFFECT OF APPLE (*MALUS DOMESTICA*) FRUIT ATTRIBUTES AND POST-HARVEST CONDITIONS ON *LISTERIA MONOCYTOGENES* SURVIVAL

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Food Science – Doctor of Philosophy

2024

ABSTRACT

Multiple recalls and outbreaks involving *Listeria monocytogenes*-contaminated apples have been linked to the post-harvest packing environment where this pathogen persists. The research presented here addresses the effect of both apple fruit attributes and post-harvest conditions on *L. monocytogenes* survival. First, *L. monocytogenes* survival was assessed on apples as affected by harvest year, apple cultivar, storage atmosphere, and growth conditions. The second portion of the study investigated the chemical composition and morphology differences of cuticular wax in Gala and Honeycrisp apples. The influence of natural cuticular wax composition and structure is important to investigate as it impacts apple fruit quality and food safety. Finally, before apple fruit are shipped to market, they are treated with commercial coatings to enhance quality. We assessed *L. monocytogenes* survival to determine the impact shellac and carnauba surface coatings have during storage in solution or applied to a contaminated surface. On the surface of apples, *L. monocytogenes* populations gradually decreased but were still quantifiable in most samples after 7 months. Apple cultivar significantly impacted *L. monocytogenes* survival ($p < 0.05$) during both harvest years with greater reductions ($p < 0.05$) seen on Gala compared to Granny Smith and Honeycrisp. Gala apples had more alkanes (g/cm2 of peel) overall for alkane compounds compared to Honeycrisp. In Gala apples the main component of the cuticular wax was nonacosane. Nonacosane content ($\mu g/cm^2$ of peel) in the cuticular waxes was greater ($p < 0.01$) on Gala (108.77±0.36) than on Honeycrisp (46.12±4.86). After just 12 hours of storage, in the shellac surface coating solution, *L. monocytogenes* was no longer detectable, compared to the carnauba surface coating solution which still supported survival (2.4-4.0 log CFU/mL) after 14

days. These findings should aid in the development of improved *L. monocytogenes* intervention strategies and inform manufacturers looking for natural food safety solutions.

ACKNOWLEDGEMENTS

I would like to thank my mentor Dr. Teresa Bergholz for her continuous support and kindness, without her I would not have completed this dissertation. Her knowledge and investigative expertise will continue to inspire me. I also wish to thank my committee members Randy Beaudry, Brad Marks, and Jeff Swada for their suggestions, guidance, and encouragement throughout my graduate career. I would also like to acknowledge Elliot Ryser for his role as the PI of the grant (WSDA SCBGP grant# K2546 & CPS Campaign for Research) that was the origin and funding for this project.

Many undergraduate and graduate students provided me with valuable support and technical assistance throughout this research. I would like to thank Carson Malhado, Gayathri Gunathilaka, Nurul Hawa Ahmad, Ian Hildebrandt, Yawei Lin, Dimple Sharma, De'Anthony Morris, Hazel Zeng, Jun Nam, Xheni Alibashi, Corrine Kamphuis, Avery Evans, and Shaney Rump.

My family and friends have provided endless support and encouragement throughout my graduate career. I would like to thank my partner Ian Diedrich for his love and encouragement throughout this process. I also wish to thank my parents Jeff and Sarah Sloniker and my sister Samantha Sloniker for being by my side throughout this entire process.

TABLE OF CONTENTS

CHAPTER 1: RATIONALE

1.1 Rationale

Up until October 2014, there had been no known foodborne outbreaks related to *Listeria monocytogenes* contamination of apples in the United States. Therefore, little research had been done up to that point on *L. monocytogenes* survival on the surface of whole, fresh apples. Most research on *L. monocytogenes* had focused on products causing most listeriosis outbreaks, soft cheeses, and deli meats. However, with increasing outbreaks of listeriosis related to fresh produce contamination at the packing house, more information was needed on the influence of apple cultivar attributes and post-harvest environmental conditions on the growth and survival of *L. monocytogenes*. Given the identified data gaps, this research investigated the following objectives and hypotheses.

Objective 1: To determine the survival of planktonic- and biofilm-derived *Listeria monocytogenes* on apples as affected by apple cultivar, storage condition, and harvest year. Hypothesis: *Listeria monocytogenes* survival will be influenced by organism growth conditions, apple cultivar, storage conditions, and harvest year.

Objective 2: To determine the composition and morphology of cuticular waxes in Gala and Honeycrisp apples.

Hypothesis: Cultivar will influence the composition and morphology of cuticular waxes.

Objective 3: To determine the impact of shellac and carnauba apple fruit surface coatings on *Listeria monocytogenes* survival.

Hypothesis: *Listeria monocytogenes* populations will decrease when exposed to apple fruit surface coatings.

The research presented here tackled these knowledge gaps and objectives. In Objective 1 *L. monocytogenes* gradually decreased but was still quantifiable in most samples after seven months. As *L. monocytogenes* was able to survive on the surface of apples for long periods, it was important to focus on possible interventions available to lower the risk of *L. monocytogenes* contamination of apples. Specifically, to further investigate possible interventions that are already present in apples or are already being used in apple processing. Objective 2 focused on the natural barrier plants have against pathogens, the cuticular wax layer. This layer is the first line of defense the apple has against *L. monocytogenes,* and this research was done to determine if the compounds present in the cuticular wax layer could point to naturally occurring antimicrobial compounds. Objective 3 focuses on surface coatings currently being applied to apples during processing and how these surface coatings may influence the survival of *L. monocytogenes*. These coatings have been used for decades and are applied to many fresh produce products, but no research had been done on how these coatings influence the survival of *L. monocytogenes,* a post-harvest contaminant responsible for numerous fresh produce outbreaks.

CHAPTER 2: LITERATURE REVIEW

2.1 Listeriosis

Listeria monocytogenes (*L. monocytogenes*) is a bacterial foodborne pathogen responsible for both isolated cases and large outbreaks of human listeriosis. In the United States, there are an estimated 1,600 illnesses and 260 deaths attributed to listeriosis annually. Over 95% of listeriosis infections lead to hospitalization resulting in a 15-20% fatality rate (Centers for Disease Control and Prevention, 2021; Food and Drug Administration, 2021). Listeriosis can present in two forms, non-invasive and invasive. The non-invasive and less severe form of listeriosis is primarily contracted by healthy individuals (Centers for Disease Control and Prevention, 2021; Food and Drug Administration, 2021). This form of listeriosis presents as a gastrointestinal illness and symptoms include fever, muscle aches, nausea, vomiting, and diarrhea. The invasive form of listeriosis is primarily contracted by at-risk populations including HIV patients, individuals under the age of 5, those over the age of 65, and people who are pregnant (Centers for Disease Control and Prevention, 2021; Food and Drug Administration, 2021). Symptoms of the invasive form of listeriosis include septicemia and meningitis caused by *L. monocytogenes* spread through the bloodstream. In people who are pregnant, listeriosis is most often associated with initial mild flu- like symptoms and fetal loss or newborn death (Centers for Disease Control and Prevention, 2021; Food and Drug Administration, 2021).

2.2 *Listeria monocytogenes*

L. monocytogenes is a heterogeneous species with 13 known serotypes, four phylogenetic lineages, as well as a growing number of clonal complexes. Of the 13 serotypes, over 95% of human cases of listeriosis have been associated with 1/2a, 1/2b, and 4b (V Ferreira et al., 2014; Orsi et al., 2011). Isolates of serotype 4b have historically caused the largest proportion of outbreaks and cases per outbreak, however in the cantaloupe outbreak of 2011 serotypes 1/2a and 1/2b were implicated in the largest and deadliest listeriosis outbreak to date in the U.S. (Centers for Disease Control and Prevention, 2021). Of the four phylogenetic lineages, I and II are associated with foodborne outbreaks while III and IV are seldom causes of human listeriosis. Additionally, serotypes 1/2b and 4b are primarily associated with lineage I and serotype 1/2a is primarily associated with lineage II (Orsi et al., 2011). Further characterization of *L. monocytogenes* groups isolates into sequence types and clonal complexes. Multilocus sequence typing (MLST) is a system that classifies *L. monocytogenes* isolates into sequence types and clonal complexes utilizing seven housekeeping genes. A clonal complex is a group of isolates that share six out of seven allelic sequences with at least one other sequence type in the group (Orsi et al., 2011).

L. monocytogenes is known for its ability to survive in unfavorable environments (Datta & Burall, 2018). Examples of these conditions include low nutrient availability, acidic pH, high osmolarity, heat shock, and other competing bacteria. In some cases, these conditions can be lethal, however, if the bacteria survive, the sublethal conditions can result in changes to the gene and protein expression of the cell. These changes can lead to resistance against further stresses, for example, cells that survived acidic conditions exhibit tolerance to subsequent exposure to heat (V

Ferreira et al., 2014). The ability of *L. monocytogenes* to adapt to repeated environmental changes makes it a continual pathogen of concern for the food industry.

2.3 *Listeria monocytogenes* outbreaks in fresh produce

Historically, food vehicles associated with *L. monocytogenes* have included processed, ready-to-eat foods including soft cheeses and sliced deli meats (Zhu et al., 2017). Conversely, in the past decade reports of *L. monocytogenes* contamination and prevalence on fresh produce have become more common. The first large *L. monocytogenes* outbreak linked to fresh produce occurred in 2011 and impacted 28 states. After consumption of cantaloupe, there were 147 patients infected, 143 hospitalized, and 33 died. One of the cases was pregnancy-related and resulted in a miscarriage (McCollum et al., 2013) This outbreak was likely due to environmental contamination as cantaloupes and environmental samples collected from the farm matched the outbreak strains (Centers for Disease Control and Prevention, 2021). The next largest *L. monocytogenes* outbreak in fresh produce occurred after consumption of enoki mushrooms spanning from 2016 to 2019. Infections were seen across 17 states in 36 people, there were 31 hospitalizations and 4 deaths. Six of the infections were pregnancy-related, and two of the infections resulted in fetal loss (Centers for Disease Control and Prevention, 2021). The third largest *L. monocytogenes* outbreak was in commercially produced, prepackaged caramel apples which will be discussed in further detail later in this review (Centers for Disease Control and Prevention, 2021).

Table 1. Confirmed outbreaks of listeriosis in the U.S. associated with produce consumption since 1979 (Centers for Disease Control and Prevention, 2021)

Year	Vehicle	State	Illnesses	Hospitalizations	Deaths
1979	Raw vegetables	MA	20	20	5
2008	Sprouts	Multistate	20	16	NA
2010	Celery	TX	10	10	5
2011	Cantaloupe	Multistate	147	143	33
2013	Frozen vegetables	Multistate	10	9	3
2014	Peaches, nectarines	Multistate	2	2	1
2014	Mung bean sprouts	Multistate	5	3	2
2014	Caramel apples	Multistate	35	34	7
2014	Sprouts	VA	2	2	0
2014	Pre-packaged leafy greens	Multistate	18	16	3
2015	Pre-packaged lettuce	Multistate	19	19	1
2016	Enoki mushrooms	Multistate	36	31	4
2016	Avocado	Multistate	10	9	1
2016	Watermelon	Multistate	6	6	2
2016	Pre-packaged salad	Multistate	10	10	1
2017	Caramel apples	Multistate	3	3	0
2017	Pre-packaged leafy greens	Multistate	10	7	3
2017	Enoki mushrooms	Multistate	5	4	1
2018	Stone fruit	Multistate	7	5	1
2021	Leafy greens	Multistate	12	12	5

2.4 *Listeria monocytogenes* incidence in fresh produce

A few recent studies have been done to determine the presence of *L. monocytogenes* in fresh produce at retail locations. A multiyear Market Basket Survey was done from 2010 to 2013 where food was purchased from retail establishments in California, Maryland, Connecticut, and Georgia (Luchansky et al., 2017). The food category with the highest percentage of *L. monocytogenes* positive samples was the raw, cut vegetable category (1.07%). In addition, the low acid cut fruits category (0.37%) exhibited higher prevalence than foods often associated with *L. monocytogenes* risk such as deli meat (0.25%), deli meat salads (0.28%), and artisanal cheese (0.16%) (Luchansky et al., 2017). Another multiyear study from 2009 to 2014 across various states assessed leafy greens, sprouts, and melons at retail for *L. monocytogenes* prevalence. Over the six years, *L. monocytogenes* was present in leafy greens (0.11%), sprouts (0.11%), and melons (0.23%) (Zhang et al., 2018).

Fresh and ready-to-eat produce products are often minimally processed and consumed raw. Fruits and vegetables are primarily grown in an outdoor environment where *L. monocytogenes* is ubiquitous (Lynch et al., 2009; Strawn et al., 2013; Zhu et al., 2017). Common pre-harvest contamination sources include irrigation water, runoff water, agricultural soil, manure, and animals (Strawn et al., 2013). While the pre-harvest environment may seem to be the obvious cause of *L. monocytogenes* produce contamination, post-harvest contamination is the primary reason for listeriosis outbreaks in fresh produce (Centers for Disease Control and Prevention, 2021).

2.5 *Listeria monocytogenes* incidence in produce processing environments

L. monocytogenes can survive in harsh conditions for a long period; therefore, it is well equipped to persist in food processing environments. During food processing, organisms may encounter sanitizers, heat treatments, and low temperatures. The ability of *L. monocytogenes* to adapt after repeated exposure to environmental stressors is why it is an ongoing post-harvest contamination concern (Strawn et al., 2013). One of the main reasons *L. monocytogenes* can persist in a harsh processing environment is its ability to join a biofilm (Colagiorgi et al., 2017). The advantages a biofilm provides to bacteria include protection from the environment, enhanced cell interaction for nutrient exchange, and horizontal transfer of antibiotic resistance genes (V Ferreira et al., 2014). While *L. monocytogenes* can form a weak biofilm on its own, it is more often associated with a multispecies biofilm. Oftentimes, *L. monocytogenes,* a poor matrix former, joins a multispecies biofilm with *Pseudomonas* spp., a good matrix former (Puga et al., 2018). A biofilm is a matrix of cells fixed to an abiotic surface that can prolong the survival of an organism. *Pseudomonas* spp. can produce a large amount of the extracellular matrix (ECM) and *L. monocytogenes,* a facultatively anaerobic organism can handle the low oxygen and nutrient availability located at the deepest layers of the mixed biofilm, therefore being less exposed to environmental stressors (H. et al., 2014; Puga et al., 2018; Sanchez-Vizuete et al., 2015; Stewart & Franklin, 2008). When food processing equipment is contaminated with a biofilm containing *L. monocytogenes* cells, contamination of food from the equipment is a major concern as cells can slough off from the biofilm and move freely in the processing environment.

Research has been done to determine the prevalence of *Listeria* species and *L. monocytogenes* in the food processing environment. *Listeria* is often used as an indicator

organism for *L. monocytogenes*, in this case, the indicator organism implies the possible presence of the pathogen. Environmental sampling was performed at 11 packing houses by collecting 1,588 samples from non-food-contact surfaces to determine the prevalence of *Listeria* during the packing season. The commodities processed at these facilities included microgreens, peaches, apples, tomatoes, broccoli, cauliflower, and cucumbers. Of the samples, 6.4% were positive for *Listeria* spp. and *L. monocytogenes*. Additionally, more than half of *Listeria* detected and isolated were confirmed *L. monocytogenes*. The sampling locations indicated that cold and wet sample sites were more likely to be positive compared to dry sites (Estrada et al., 2020).

2.6 *Listeria monocytogene*s recalls and outbreaks in apples

The first recalls for apples contaminated with *Listeria monocytogenes* were issued in diced and sliced apples in 2012 and 2013 (Food and Drug Administration, 2021). In October 2014 apples were first identified in a listeriosis outbreak. Concerns were raised in October 2014 when consumption of caramel apples was linked to 35 cases of listeriosis across 12 states, including 34 hospitalizations, and one death (Buchanan, 2016; Centers for Disease Control and Prevention, 2021). Environmental testing along the supply chain found two *L. monocytogenes* strains in the apple-packing facility that matched the two clinical isolates. Further characterization of the strains confirmed they were both serotype 4b1 but belonged to different sequence types (ST) and clonal groups (CC), ST1/CC1 and ST338/CC382 (Angelo et al., 2017; Chen et al., 2016; Colagiorgi et al., 2017; Food and Drug Administration, 2021; Garner & Kathariou, 2016; Gorski et al., 2022). A second caramel apple outbreak of listeriosis was reported in October 2017, resulting in three hospitalizations. Although finished product and environmental samples were collected along the supply chain, the only *L. monocytogenes* isolate recovered was a non-outbreak-related strain from the supplier (Marus et al., 2019). In December 2017, whole apples and packaged apple slices were recalled from five states due to *L. monocytogenes* contamination. This recall was followed by two others in 2019 and 2020 that involved whole and sliced apples contaminated during processing and packing (Food and Drug Administration, 2021).

2.7 *Listeria monocytogenes* survival on apples

Fresh apples are sold in a variety of ways to consumers including whole, sliced, diced, and caramel or candy-coated. *L. monocytogenes* recalls and outbreaks have been associated with all variations of commercially available apples therefore, there is some food safety research that has been done to determine the survival of *L. monocytogenes* on whole, sliced, diced, and caramel or candy-coated apples.

In a study on caramel and non-caramel-coated whole apples inoculated with a high *L. monocytogenes* inoculation level (7 log/CFU) on the stem end and the equatorial surface. The stem end supported cell growth whereas the equatorial surface maintained cell survival but no growth. Additionally, at a lower *L. monocytogenes* inoculation level (3 log/CFU), the stem end and the equatorial surface both supported survival but not growth on fresh apples. However, caramel apples inoculated at the stem end did exhibit significant growth (Salazar et al., 2016). Additionally, the core of a caramel apple has been found to internalize *L. monocytogenes* due to stick insertion, resulting in growth during storage at varying temperatures (Glass et al., 2015; Gustafson, 2017).

Regarding sliced apples, *L. monocytogenes* populations were found to increase on sliced apples at temperatures between 10-25 °C (Alegre et al., 2010; Conway et al., 2000). On whole apples, a study was performed to determine the impact of storage temperature and apple cultivar on *L. monocytogenes* survival. Two inoculation levels were used, 3.5 and 6 log CFU/apple, and apples were stored under 1, 4, 10, and 22 °C for up to 3 months. During storage, a reduction of 0.5-1.5 log CFU/apple for *L. monocytogenes* was observed. Both inoculation levels had similar results and Granny Smith apples stored at 1, 4, or 10 °C had a lower reduction of *L. monocytogenes* compared to 22°C (Sheng et al., 2017). In 2021, a study

was conducted to determine the impact of long-term low-temperature sea freight from New Zealand to the US and Europe by simulating 0.5°C and 20 °C temperatures for storage (Nangul et al., 2021). There was no significant difference in survival of *L. monocytogenes* log CFU/apple between the storage temperatures and *L. monocytogenes* populations were higher in the calyx compared to the equatorial skin after the simulation (Nangul et al., 2021). These multiple studies have shown that *L. monocytogenes* survives on the apple surface regardless of inoculation level, apple cultivar, storage temperature, or storage length.

2.8 *Listeria monocytogenes* survival in apple processing environments

The 2014-15 caramel apple outbreak confirmed environmental contamination was the cause of the outbreak. Since the outbreak, studies have been done to determine the presence of *L. monocytogenes* on apple processing facility food and non-food contact surfaces. For one study on tree fruit packing houses, samples were taken from non-food-contact surfaces in cold storage rooms and on packaging lines. Of the samples, 17.5% of them were positive for *L. monocytogenes*. Samples were also taken throughout the apple packing season. *L. monocytogenes* prevalence occurred as the packing season progressed from fall, peaking during winter, and decreasing during spring. The sample locations with the highest prevalence were the processing line area, cold storage, and packaging lines, respectively (Simonetti et al., 2021).

Food contact surfaces were sampled in five apple packing houses for two packing seasons. In total, 2,988 samples were taken from 50 sites, and 4.6% were positive for *Listeria* spp. The location in the packinghouses that *Listeria* spp. was most frequently isolated from the wax coating unit operations (Ruiz-Llacsahuanga et al., 2021). From November 2017 – April 2018 environmental samples were taken from three tree fruit packing facilities. Each facility had 39 samples collected and *L. monocytogenes* was confirmed in 11, 39, and 16 samples from facilities F1, F2, and F3, respectively (Tan et al., 2019). In 2020, an assessment was published on the attitudes of apple packers on food safety and when asked to rate food safety topics in priority of training for apple packers *Listeria* ranked first with a score of 2.5, 1 representing low priority and 3 representing high priority (Atis, 2020).

2.9 Apple processing

Apple fruit goes through multiple processing steps after being harvested and before market arrival. First, the apples are transported to a packing house or cold storage facility from the orchard or previous storage location. Apples are transported and stored in large wooden bins, the storage conditions are air or controlled atmosphere conditions, 1-4% oxygen and 0-2% carbon dioxide, at 0-2 °C with a relative humidity of 90-95% (Ruiz-Llacsahuanga et al., 2021; Tripu & Farcuh, 2021). During storage, apples are typically treated with 1-methylcyclopropene (1-MCP) to slow the ripening process. At a target concentration of one part per million and just one exposure, 1-MCP slows the rise in ethylene and respiration, aroma production, and softening (Beaudry & Watkins, 2003; Tripu & Farcuh, 2021). After air storage for up to three months or controlled atmosphere storage for up to 12 months, apples are removed from storage in their bins and dumped or floated into flumes of water. Following water washing and separation of leaves and damaged fruit, the apples are treated with soaps and/or sanitizers on brush beds. Common sanitizers include chlorine and peracetic acid (Wang et al., 2023). The apples are then sprayed with shellac or carnauba surface coatings as apples roll on a conveyor. After apples are coated, they go through a tunnel drying step. The temperature of the air for unwaxed and waxed fruit is typically 30 °C and 50 °C, respectively. Apple fruit is then sorted for size, quality, and color before being packed and labeled. The packaged fruit is typically shipped to market that same day or kept in cold storage until shipping.

2.10 Apple surface coating unit operations

The apple surface coating unit operation has been designated a hotspot for *Listeria monocytogenes* during environmental sampling studies (Estrada et al., 2020; Ruiz-Llacsahuanga et al., 2021; Simonetti et al., 2021). A research group analyzed 32 studies to determine areas of concern for persistent *Listeria* in produce operation facilities. The key areas in the facilities that required intervention were forklifts and the produce waxing unit operation (Belias, 2021). The location in the packinghouses that *Listeria* spp. is most frequently isolated from is the wax coating unit operations (Ruiz-Llacsahuanga et al., 2021). These research findings parallel conversations conducted with apple industry members who highlighted the apple wax unit operation as a concern area for *Listeria* spp. The surface coating unit operation is difficult to clean, and if the coating material is not removed, it can build up and trap organisms leading to ineffective sanitation. Even though apple packing facilities use sanitizers like chlorine and peracetic acid (PAA) as a method for pathogen prevention, the surface coating unit operation, which is after sanitization in the processing line is still a hot spot for *Listeria*.

2.11 Natural apple surface coating

The surface of an apple is made up of a naturally formed protective layer called the cuticle. The cuticle protects the fruit by preventing water loss, shielding against mechanical damage and harmful irradiation, as well as providing a line of defense against insects, fungi, and pathogenic microorganisms (Dominguez et al., 2017; Serrano et al., 2014). The apple fruit cuticle is interspersed with epicuticular and intracuticular waxes. These waxes primarily consist of fatty acids, aldehydes, primary and secondary alcohols, ketones, alkanes, and alkyl esters. The composition of the cuticular waxes is influential on the morphology, arrangement, and microstructures of the plant surface, which can then influence adherence to water, pesticides, fungal spores, and microbial pathogens (Belding et al., 2000; Burnett et al., 2000; Serrano et al., 2014).

2.12 Commercial apple surface coatings

Surface coatings are a thin, edible layer of material applied to a product surface. Lipid-based coatings are most common for apples and are mainly used for their hydrophobic properties (Dhall, 2013; Pashova, 2023). The coating provides a barrier against water loss, reduces respiration, and improves the appearance of the fruit. The two most common lipid coatings shellac and carnauba are wax and resin-based. Shellac resin is secreted by the female lac bug (*Laccifer lacca*) and is made up of a mixture of long-chain esters of monovalent alcohols and acids. Shellac is primarily used for its glossy sheen in coatings for the food, cosmetics, pharmaceuticals, and furniture (Dhall, 2013). Carnauba wax is present on the surface of Brazilian palm (*Coernicia cerifera*) leaves and is made up of long-chain alcohols and esters. Carnauba wax has a high melting point and is one of the hardest plant waxes often used to create a humidity barrier. Applications of carnauba wax include coatings for food, cosmetics, automobiles, and furniture (Pashova, 2023).

While edible coatings have been found to enhance the quality and extend shelf-life of apples; limited research has been done on the impact of these commonly used commercial wax coatings on foodborne pathogens associated with food applications (Raghav et al., 2016). One study tested the impact of different shellac formulations on the survival of *Escherichia coli*, *Enterobacter aerogenes*, and *Klebsiella pneumonia* in liquid solution (McGuire & Hagenmaier, 2001). A shellac coating solution with 5% morpholine reduced *Escherichia coli* and *Klebsiella pneumonia* from 6 log CFU/mL to below the limit of detection (5 CFU/mL) in three hours at 24°C (McGuire & Hagenmaier, 2001). In a study on novel natural composite films, a disk diffusion method was used to determine the impact of varying amounts of shellac on the survival of *Bacillus mycoides*, *Escherichia coli*, and *Candida albicans (Mohamed et al.*,

2019). They found the inhibition zones increased in size as the amount of shellac increased in the composite films. For the 8% shellac films, there was a 12 mm inhibition zone for *Escherichia coli*, and in the 14% shellac films, *Escherichia coli*, *Bacillus mycoides*, and *Candida albicans*, had inhibition zones 17, 10, and 10 mm, respectively (Mohamed et al., 2019). In a study on the effect of a shellac surface coating on *L. monocytogenes* survival on apples, after two months of storage, apples that had been coated with shellac had significantly ($p < 0.05$) more survival compared to apples that had not been treated with wax (Macarisin et al., 2019). However, for Red Delicious and Fuji apples, the *L. monocytogenes* populations in uncoated apples were significantly ($p < 0.05$) higher compared to the coated apples for 30 days. Typically, when apples are removed from storage, they are processed, and shipped to the market the same day. Based on this timeline, the short-term impact of surface coatings on *L. monocytogenes* reduction is most critical to food safety.

CHAPTER 3: FATE OF PLANKTONIC AND BIOFILM-DERIVED *LISTERIA MONOCYTOGENES* ON UNWAXED APPLES DURING AIR AND CONTROLLED ATMOSPHERE STORAGE

Sloniker, N., Raftopoulou, O., Chen, Y., Ryser, E. T., Beaudry, R. (2023). Fate of Planktonic and Biofilm-Derived *Listeria monocytogenes* on Unwaxed Apples during Air and Controlled Atmosphere Storage. *Foods*, *12*(19). https://doi.org/10.3390/foods12193673

3.1 Abstract

Multiple recalls and outbreaks involving *Listeria monocytogenes*-contaminated apples have been linked to the post-harvest packing environment where this pathogen can persist in biofilms. Therefore, this study assessed *L. monocytogenes* survival on apples as affected by harvest year, apple cultivar, storage atmosphere, and growth conditions. Unwaxed Gala, Granny Smith, and Honeycrisp apples were dip-inoculated in an 8-strain *L. monocytogenes* cocktail of planktonic- or biofilm-grown cells (~6.5 log CFU/mL), dried, and then examined for numbers of *L. monocytogenes* during air or controlled atmosphere (CA) (1.5% O_2, 1.5% CO_2) storage at 2 °C. After 90 days, air or CA storage yielded similar *L. monocytogenes* survival ($p > 0.05$), regardless of harvest year. Populations gradually decreased with *L. monocytogenes* quantifiable in most samples after 7 months. Apple cultivar significantly impacted *L. monocytogenes* survival ($p < 0.05$) during both harvest years with greater reductions ($p < 0.05$) seen on Gala compared to Granny Smith and Honeycrisp. Biofilm-derived cells survived longer ($p < 0.05$) on *L. monocytogenes*-inoculated Gala and Honeycrisp apples compared to cells grown planktonically. These findings should aid in the development of improved *L. monocytogenes* intervention strategies for apple growers and packers.

3.2 Introduction

Listeria monocytogenes (*L. monocytogenes*) is a Gram-positive bacterial foodborne pathogen that causes an estimated 1600 illnesses and 260 deaths annually in the United States alone. The populations most susceptible to listeriosis include newborns and infants, the elderly, pregnant women, and immunocompromised individuals (Centers for Disease Control and Prevention, 2021). For these populations, *L. monocytogenes* can spread through the bloodstream and cause septicemia and meningitis. Over 95% of listeriosis infections lead to hospitalization, with a fatality rate of 15–20% (Centers for Disease Control and Prevention, 2021; Datta & Burall, 2018). In pregnant women, listeriosis is most often associated with fetal loss or death of the newborn infant (Centers for Disease Control and Prevention, 2021). The vast majority of listeriosis outbreaks are caused by just three *L. monocytogenes* serotypes, 1/2a, 1/2b, and 4b (Desai et al., 2019). Historically, food vehicles associated with *L. monocytogenes* have included unpasteurized dairy products, soft cheeses, and sliced deli meats (Desai et al., 2019). In the past decade, *L. monocytogenes* outbreaks have increasingly become associated with foods considered to be "moderate risk" or "low risk," including stone fruit and caramel apples (Buchanan et al., 2016; Desai et al., 2019).

Apples and apple-derived products have been well documented as vehicles of foodborne illness. In 1997, outbreaks of gastrointestinal illness and hemolytic uremic syndrome caused by *Escherichia coli* O157:H7 were linked to unpasteurized apple juice and apple cider (Centers for Disease Control and Prevention, 1997). These outbreaks raised concerns related to pathogen growth and survival on apples. After dip-inoculating apples with *E. coli* O157:H7, Buchanan et al. found the highest populations in the stem and calyx portions, which can entrap contaminants, providing a favorable microenvironment for microbial growth (Buchanan et al.,

1999). Conway et al. subsequently reported the growth of *L. monocytogenes* on sliced apples during air and controlled atmosphere at 10 and 20 °C (Conway et al., 2000). In 2012 and 2013, the first recalls were issued for *L. monocytogenes*-contaminated diced and sliced apples that were distributed across 36 states. However, at that time, apples had not been identified as a listeriosis risk due to the low acid (pH < 4.0) of the fruit.

New concerns were raised in October 2014 when consumption of *L. monocytogenes*-contaminated caramel apples was traced to 35 cases of listeriosis across 12 states, including 34 hospitalizations and one fatality (Buchanan et al., 2016). Environmental testing along the supply chain identified two *L. monocytogenes* serotype 4b strains in the apple-packing facility that matched two clinical isolates (Angelo et al., 2017; Colagiorgi et al., 2017; Food and Drug Administration, 2021; Garner & Kathariou, 2016). A second caramel apple outbreak of listeriosis was reported in October 2017, which resulted in three hospitalizations. Although finished product and environmental samples were collected along the supply chain, the only *L. monocytogenes* isolate recovered was a non-outbreak-related strain from the supplier (Marus et al., 2019). In December 2017, whole apples and later packaged products containing apple slices were recalled from five states due to *L. monocytogenes* contamination (Food and Drug Administration, 2021). This recall was followed by two others in 2019 and 2020, involving whole and sliced apples contaminated during processing and packing. Taken together, these recalls and outbreaks reinforce the need to understand better the potential harborage sites in apple packing facilities and the subsequent persistence of *L. monocytogenes* on apples (Food and Drug Administration, 2021).

L. monocytogenes is a frequent environmental contaminant of apple packinghouses where it can form biofilms on product contact surfaces, including polishing brushes, roller

conveyors, dividers, and brushes under fans and blowers (V Ferreira et al., 2014; Kaminski et al., 2014; Macarisin et al., 2019; Pietrysiak et al., 2019; Ruiz-Llacsahuanga et al., 2021; Scollon et al., 2016; Srey et al., 2013; Tan et al., 2019; Zhu et al., 2017). Biofilm formation begins with the attachment of cells to a surface, followed by bacterial growth and the production of extracellular polymeric substances. Biofilm formation is impacted by temperature, surface material, and nutrient availability. Once mature, biofilms allow for the flow of nutrients and waste between the cells as well as the release of cells that can contaminate the product or form additional biofilms on other food contact surfaces (Pietrysiak et al., 2019). Protected from chemical sanitizers and other forms of environmental stress, *L. monocytogenes* can survive within these biofilms for months or years (Botticella et al., 2013; Garner & Kathariou, 2016; Rodriguez-Lopez et al., 2018; Yu & Chen, 2019; Zhu et al., 2017). Identical *L. monocytogenes* genotypes have been recovered from packinghouses and patients in other outbreaks involving diced celery (2010), whole cantaloupe (2011), stone fruit (2014), and mung bean sprouts (2014), confirming the long-term persistence of *L. monocytogenes* in packinghouse environments (Garner & Kathariou, 2016).

Several studies have assessed the survival of planktonically grown cells of *L. monocytogenes* on different apple cultivars during storage. When Sheng et al. inoculated unwaxed Fuji and Granny Smith apples with a cocktail of *L. monocytogenes* strains to contain either 3.5 or 6.0 log CFU/apple, populations decreased 0.5–3.0 logs during 3 months of storage at 1–10 °C (Sheng et al., 2017). Limited *L. monocytogenes* reductions have also been reported by others on whole apples after 3 to 5 months of cold storage (Macarisin et al., 2019; Nangul et al., 2021; Sheng et al., 2017). However, none of these studies assessed the viability of biofilm-

grown cells of *L. monocytogenes* on apples during long-term storage, multiple harvest seasons, different apple growing regions, or storage room atmosphere.

Given the identified data gaps, this study investigated *L. monocytogenes* survival on apples as impacted by (1) apple cultivar (Gala, Granny Smith, and Honeycrisp), (2) harvest season, (3) storage conditions (air or CA), and (4) the type of inoculum (planktonic- or biofilm-grown cultures) to better mimic the contamination from water or food contact surfaces in apple packinghouses.

3.3 Materials and Methods

Apples and Storage Conditions. Unwaxed Gala, Granny Smith, and Honeycrisp apples (6–8 cm dia.) were shipped from three major apple-growing regions in the United States (Midwest, Northeast, and Northwest) to Michigan State University during the 2019 and 2020 harvest seasons. Upon arrival, any damaged or under-sized apples were discarded. The remaining apples were stored in 0.93 m^3 aluminum chambers (Storage Control Systems, Sparta, MI) under air or controlled atmosphere (CA) (1.5% O_2, 1.5% CO_2) in a 2 °C cold room and treated within 7 d of arrival with 1 μL/L 1-methylcyclopropene for 24 h to suppress ripening and preserve fruit quality. The chamber atmosphere was regulated by an automated atmosphere control system (ICA 61 Laboratory System: International Controlled Atmosphere Ltd., Paddock Wood, UK), with the temperature monitored continuously.

Bacterial Strains and Growth Conditions. Eight *L. monocytogenes* strains belonging to serotypes 1/2a, 1/2b, and 4b came from the laboratory of Dr. Sophia Kathariou at North Carolina State University. The panel included two different strains from the 2014–2015 caramel apple outbreak as well as strains from several other listeriosis outbreaks (Table 1). The strains were barcoded for subsequent metagenomic analysis with unique 30-bp DNA sequences constructed and incorporated into the chromosome as previously described (Zhang et al., 2017). Plasmid pTZ200.mix—a derivative of the pPL2 plasmid that allows stable incorporation of the barcodes into the chromosome—was extracted from *Escherichia coli* SM10λpir and electroporated into each of the strains (Lauer et al., 2002; Zhang et al., 2017). Transformants were selected on Brain Heart Infusion (BHI) agar containing chloramphenicol (plasmid marker; 10 μg/mL). Individual colonies were analyzed by PCR and Sanger sequencing to identify uniquely barcoded isolates. For one of the strains, 4b1, barcoding was

based on chromosomal *gfp* sequences (Li, 2001). Whole genome sequences were obtained for all strains and their parental counterparts. The strains were stored at -80 °C in trypticase soy broth containing 0.6% (*w*/*v*) yeast extract (TSBYE, Neogen, Lansing, MI, USA) and 10% (*v*/*v*) glycerol (Sigma-Aldrich, Inc., St. Louis, MO, USA). Working cultures were prepared by streaking the frozen stock culture onto modified tryptic soy agar (mTSAYE) containing 0.6% yeast extract (Neogen), 0.1% (*w*/*v*) esculin, and 0.5% (*w*/*v*) ferric ammonium citrate (Sigma-Aldrich) followed by incubation at 37 °C for 48 h. Each of the barcoded strains was compared to its parental strain for biofilm formation, hemolytic activity, motility on soft agar, and virulence using the *Galleria mellonella* model as previously described (Parsons et al., 2017). Based on these test results, genetic barcoding did not affect the strain phenotypes.

Table 2. Panel of eight barcoded *Listeria monocytogenes* strains

Strain	**Serotype**	**Genotype**	**Outbreak**
4b1-GFP	4b	ST2	Clinical isolate,1962
F2365-2	4b	ST1	California cheese outbreak, 1985
H7858-1	4b	ST6	Hot dog outbreak, 1998–99
2010L-1723-4	1/2a	ST378	Celery outbreak, 2010
CFSAN023957-A10	4bv-1	ST554	Mung bean sprouts outbreak, 2014
2014L-6680-7	4b	ST1	Caramel Apple outbreak, 2014–2015
2014L-6695-5	4b	ST382	Caramel Apple outbreak, 2014–2015
CFSAN073872-6	1/2b	ST581	Apples, 2017

Preparation of Planktonic and Biofilm-derived L. monocytogenes Inoculum. The eight *L. monocytogenes* strains were separately grown (37 °C, 24 h) in TSBYE. After adjusting the OD_{600} values to 0.600–0.650, the cultures were combined in equal volumes, pelleted twice by centrifugation (9000× *g*, 15 min, 4 °C), resuspended in phosphate-buffered saline (PBS) and diluted to ~10^7 CFU/mL in 1.6 L of deionized (DI) water for apple inoculation. *L. monocytogenes* populations in the inoculum were confirmed by plating appropriate dilutions on mTSAYE and Modified Oxford agar (MOX, Neogen) followed by incubation at 37 °C for 36–48 h. For the biofilm inoculum, the same strains were similarly grown (37 °C, 24 h) in TSBYE, adjusted to the same OD_{600} range, and combined in equal proportions to obtain an 8-strain cocktail. Thereafter, 400 μL of the cocktail was added to each of 80 150 mm-dia. Petri plates followed by 19.6 mL of tryptic soy broth (TSB). After incubation (37 °C, 48 h), the TSB was discarded, and the plates were rinsed twice with 1 mL of PBS. Biofilm cells were harvested from the plates using three sterile PBS-moistened cotton-tipped swabs (Puritan, Guilford, ME, USA) per plate, which were transferred to a sterile 50 mL Corning polypropylene centrifuge tube (ThermoFisher Scientific, Waltham, MA, USA) containing 20 mL of PBS and vortexed for 1 min. After removing the swabs, these 80 biofilm-derived suspensions were combined to obtain the cocktail as described above for planktonic cells and then diluted to ~10^7 CFU/mL in 1.6 L of deionized (DI) water for apple inoculation.

Apple Inoculation and Storage. The planktonic and biofilm-derived inoculums (1.6 L) were each added to 14.4 L of DI water in a 56.8 L Nalgene™ Lightweight Graduated Cylindrical Tank (ThermoFisher Scientific) lined with low-density polyethylene bags (ULINE, Pleasant Prairie, WI, USA) to obtain a population of ~ 6.5 log CFU/mL.

Groups of ~15 apples were transferred to mesh produce bags (Product Packaging Supplies, Elgin, IL, USA) and initially washed in 5 L of DI water to remove any soil or debris, with the water changed after every six bags. Thereafter, duplicate bags of apples were immersed in 16 L of the *L. monocytogenes* cocktail and continuously agitated for 10 min using a sanitized plastic pole (ULINE, Pleasant Prairie, WI, USA). After draining, the apples were aseptically removed from the bags, dried at room temperature on an aluminum foil-lined shelf while periodically turning to prevent pooling of the inoculum in the stem bowl or calyx, and finally transferred to the chambers described above for storage at 2 °C in air or CA (1.5% O_2, 1.5% CO_2).

Sampling and L. monocytogenes Enumeration. Inoculated apples were sampled immediately after air-drying (day 0), weekly during the first month, and then monthly. Two composite samples of three apples each were randomly removed from storage at each time point. After removing the stem bowl and calyx portions with a sterile knife, the remaining skin was removed using an electric apple peeler (Rotato Express, Electric Peeler 093209-006-BLCK, Starfrit, QC, Canada). The stem and calyx portions were added to one Whirl-pak bag (Nasco, Modesto, CA, USA), and the peel was added to a second Whirl-pak bag. The samples were diluted 1:5 in sterile PBS (*w*/*v*) and then homogenized in a stomacher (Stomacher 400 Circulator, Seward, Worthington, UK) for 1 min at 300 rpm. Appropriate PBS dilutions were plated on mTSAYE and MOX with *L. monocytogenes* colonies enumerated after 48 h of incubation at 37 °C. After just 30 days of storage, counts for the peel alone were typically near the limit of detection. Therefore, the counts from the stem/calyx and the peel were combined and expressed as log CFU/apple.

Statistical Analysis. Two independent apple storage trials were conducted in the fall of 2019 and fall of 2020. Two composite samples of three apples each per cultivar and growing region were analyzed at each sampling time. Analysis of covariance (ANCOVA) and post-hoc pairwise comparisons were used to determine statistical significance at $p \leq 0.05$ using JASP software version 0.14.1 (The JASP Team, Amsterdam, The Netherlands). Response variables analyzed include storage atmosphere, harvest year, apple cultivar, and inoculum type. Box plots were created using RStudio Professional Version 2022.07.0 (RStudio, Inc., Boston, MA, USA).

3.4 Results

Dip inoculation in the planktonic and biofilm suspensions yielded *L. monocytogenes* populations of 3.22–5.50 and 5.97–6.60 log CFU/apple, respectively, with no significant differences seen between cultivars. Overall, *L. monocytogenes* populations decreased over time; however, the pathogen was still quantifiable at ~2 to 4 log CFU/apple in most samples after 7 months of storage. Some planktonic- and biofilm-inoculated apples still yielded *L. monocytogenes* populations of 4.6 log CFU/apple after 210 days of CA storage.

Storage Atmosphere. After 90 days of storage at 2 °C, no significant difference ($p > 0.05$) in *L. monocytogenes* survival was observed between the apples subjected to air or CA storage, regardless of harvest year (Figure 1). *L. monocytogenes* populations on Gala and Honeycrisp apples from harvest year 1 decreased ~0 to 3.5 log CFU/apple after 90 days of air and CA storage. However, *L. monocytogenes* was more persistent on Granny Smith apples, decreasing < 1.0 log CFU/apple during 30 to 90 days of air and CA storage. *L. monocytogenes* populations on Gala and Granny Smith apples from harvest year 2 initially decreased ~2 to 2.5 log CFU/apple during 14 to 90 days of air and CA storage, whereas populations on Honeycrisp apples initially decreased ~2.0 log CFU/apple between 30 and 90 days of storage (Figure 1).

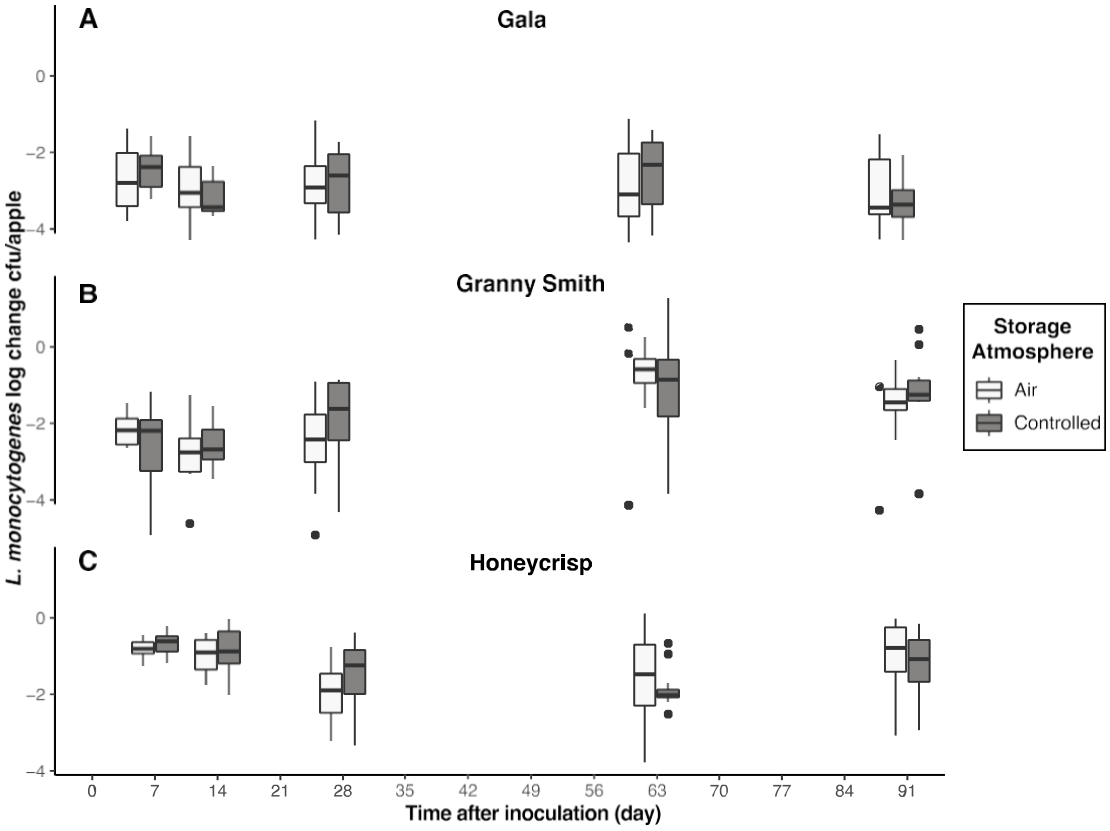

Figure 1. Reductions of *L. monocytogenes* populations on inoculated apples. Reductions were determined as log CFU/apple and shown as boxplots. Unwaxed Gala (**A**), Granny Smith (**B**), and Honeycrisp (**C**) apples were inoculated with cocktails of planktonic cultures and stored in air and controlled atmosphere storage at 2 °C.

Harvest Year. *L. monocytogenes* persistence was significantly ($p < 0.05$) impacted by harvest year, with greater overall survival observed in harvest year 1 (Figure 2). However, decreased survival of *L. monocytogenes* was observed on Granny Smith apples from harvest year 1 ($p < 0.05$) compared to harvest year 2. After 210 days of storage, *L. monocytogenes* populations on Granny Smith apples decreased <0.64 and 1.40–3.85 log CFU/apple for harvest years 1 and 2, respectively, compared to 1.20–3.15 and 0.93–3.05 log for Honeycrisp apples (Figure 2).

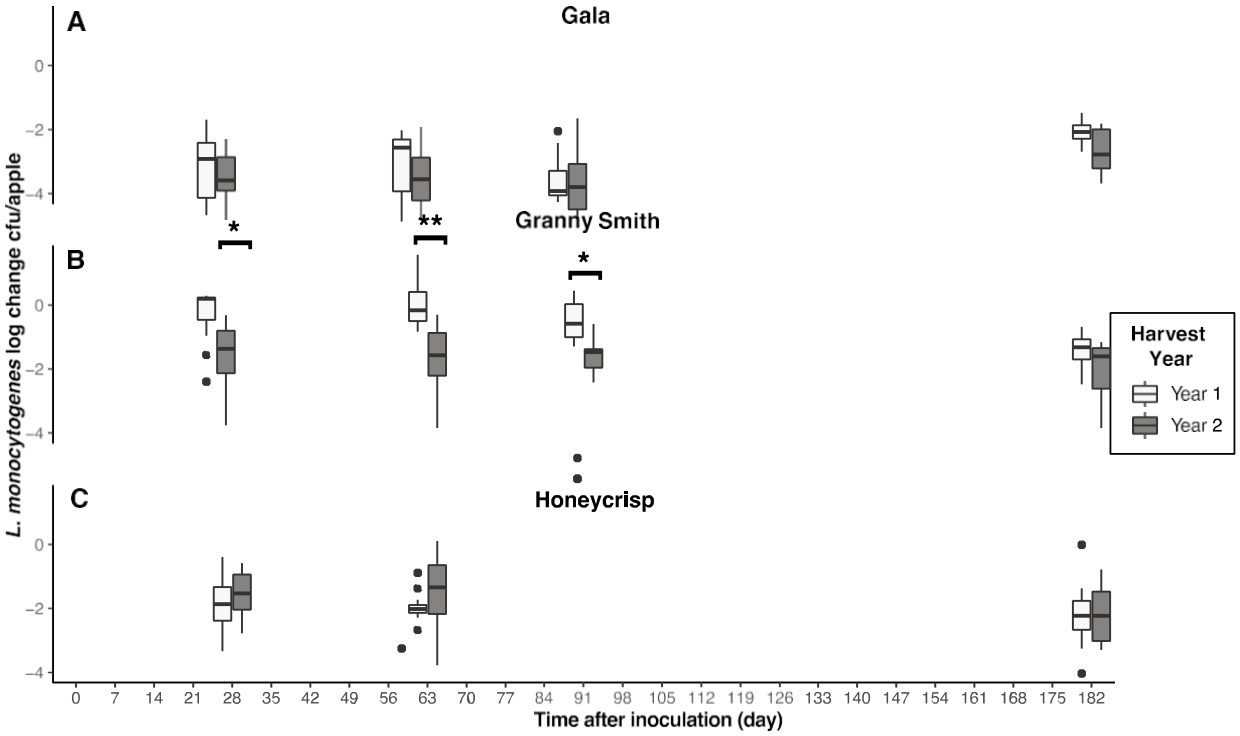

Figure 2. Reductions of *L. monocytogenes* populations on inoculated apples. Reductions were determined as log CFU/apple and shown as boxplots. The asterisk (*) is used to represent a statistically significant result. Unwaxed Gala (**A**), Granny Smith (**B**), and Honeycrisp (**C**) apples from harvest year 1 and harvest year 2 were inoculated with cocktails of planktonic cultures and stored at 2 °C.

Apple Cultivar. *L. monocytogenes* survival was significantly ($p < 0.05$) impacted by apple cultivar during both harvest years (Figure 2). However, regardless of harvest year, significantly ($p < 0.05$) greater reductions in *L. monocytogenes* were seen for Gala compared to Granny Smith and Honeycrisp apples. After 210 days of storage, *L. monocytogenes* populations on Gala apples decreased from 0.34 to 2.29 and 1.85 to 3.43 log CFU/apple for harvest years 1 and 2, respectively (Figure 2).

Inoculum Type. Regardless of harvest year, *L. monocytogenes* survival on Granny Smith apples was not significantly ($p > 0.05$) impacted by the type of inoculum (Figures 3 and 4). However, inoculum type did significantly ($p < 0.05$) impact *L. monocytogenes* survival on Gala and Honeycrisp apples. Biofilm-grown cells of *L. monocytogenes* survived significantly ($p < 0.05$) longer than planktonically grown cells on Gala apples for both harvest years and for Honeycrisp apples in harvest year 1 (Figures 3 and 4). After 210 days of storage, *L. monocytogenes* populations on Gala apples inoculated with planktonically grown cells decreased 2.02 and 3.43 log CFU/apple for harvest years 1 and 2, respectively, as compared to 0.56 and 2.18 log CFU/apple for biofilm- grown cells. Numbers of planktonically grown cells on Honeycrisp apples from harvest years 1 and 2 decreased 2.55 and 1.16 log CFU/apple after 210 days of storage, respectively, compared to 0.01 and 2.76 log CFU/apple for biofilm-grown cells.

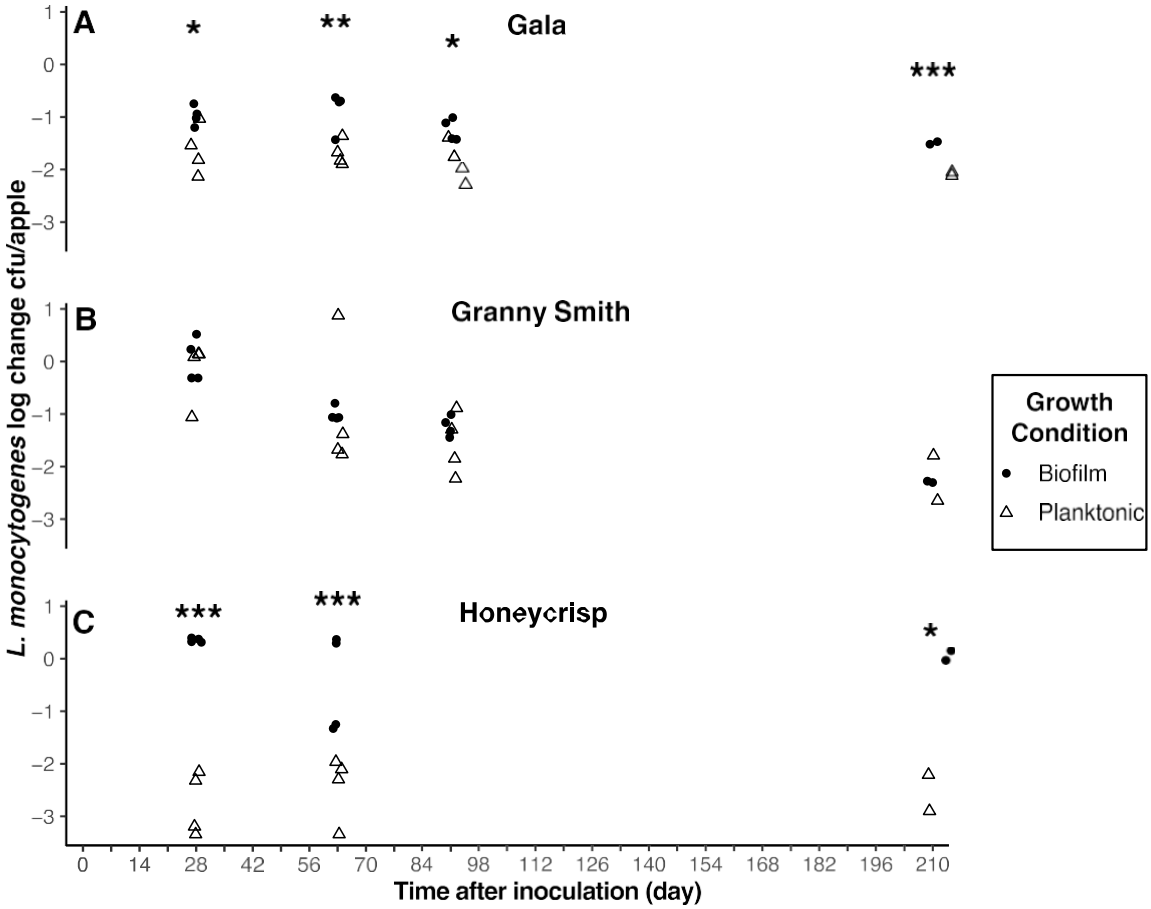

Figure 3. Reductions of *L. monocytogenes* populations on inoculated apples. Reductions were determined as log CFU/apple and shown as scatterplots. The asterisk (*, **, ***) is used to represent a statistically significant ($p < 0.05$, 0.01, 0.001) result. Unwaxed Gala (**A**), Granny Smith (**B**), and Honeycrisp (**C**) apples from harvest year 1 were inoculated with cocktails of biofilm (black circle) and planktonic (white triangle) cultures and stored at 2 °C.

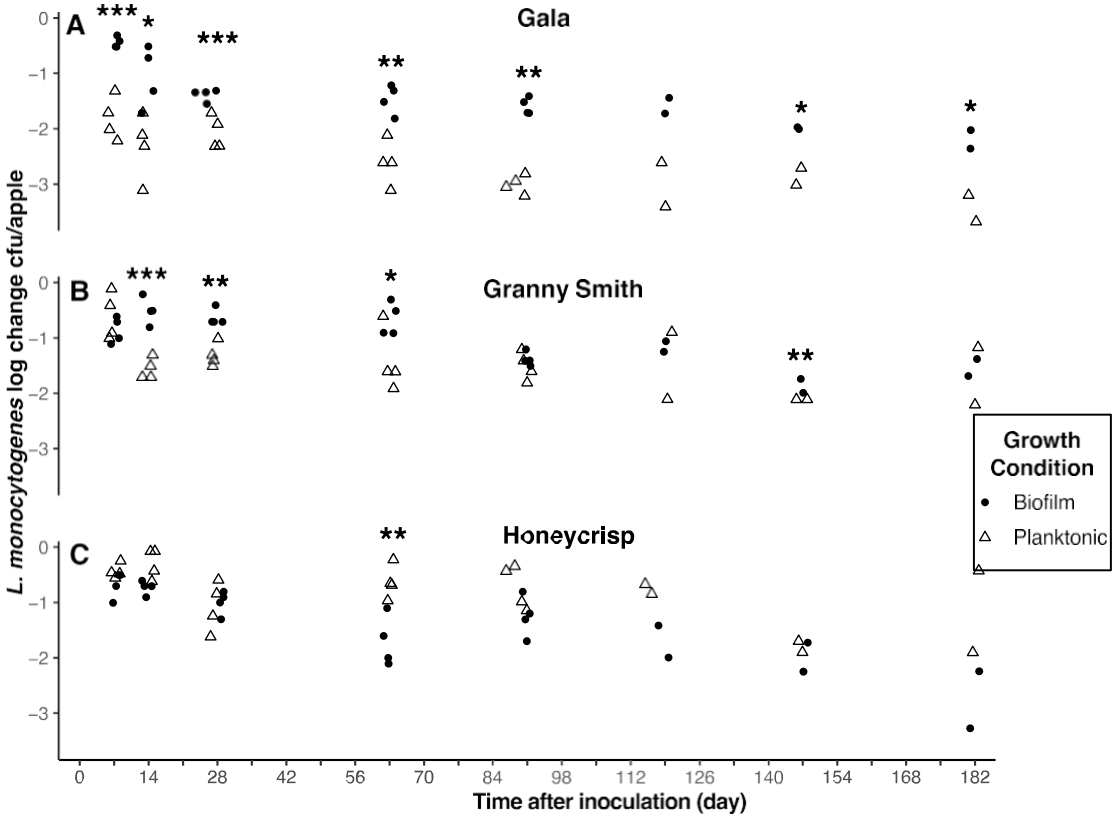

Figure 4. Reductions of *L. monocytogenes* populations on inoculated apples. Reductions were determined as log CFU/apple and shown as scatterplots. The asterisk (*, **, ***) is used to represent a statistically significant ($p < 0.05$, 0.01, 0.001) result. Unwaxed Gala (**A**), Granny Smith (**B**), and Honeycrisp (**C**) apples from harvest year 2 were inoculated with cocktails of biofilm (black circle) and planktonic (white triangle) cultures and stored at 2 °C.

3.5 Discussion

In this study, the survival of *L. monocytogenes*, which is a facultative anaerobe, was similar between air and CA storage after 90 days ($p > 0.05$), regardless of harvest year. Greater *L. monocytogenes* survival was seen in year 1 compared to year 2 ($p > 0.05$). Apple cultivar significantly impacted *L. monocytogenes* survival ($p > 0.05$) during both harvest years, with greater reductions ($p > 0.05$) seen on Gala compared to Granny Smith and Honeycrisp. Biofilm-derived cells survived longer ($p > 0.05$) on *L. monocytogenes*-inoculated Gala and Honeycrisp apples compared to cells grown planktonically.

Air and CA storage supported similar survival of *L. monocytogenes* on all three apple cultivars. Our findings are supported by Scollard et al., who observed no significant differences ($p > 0.05$) in *L. monocytogenes* survival when a model vegetable system was stored in air and CA (5 and 20% CO_2) (Scollard et al., 2009). After harvest, apples are held refrigerated in either air or a controlled atmosphere, which will alter the apple microbiome, decrease the apple respiration rate, and extend shelf life during storage (Wright et al., 2015; Yu & Chen, 2019). Decreased oxygen levels may also be responsible for enhancing resistance to various environmental stressors (Jydegaard-Axelsen et al., 2004; Lungu et al., 2009; Roberts et al., 2020).

As previously mentioned, greater *L. monocytogenes* survival was observed in harvest year 1 compared to harvest year 2 ($p > 0.05$). In a study by Bösch et al. that assessed the changing microbiome of apples harvested during 2015–2018, harvest year was one of the two top contributors to both the numbers and diversity of bacteria and fungi, including *Botrytis*, *Monilinia*, *Neofabraea*, and *Penicillium* (Bosch et al., 2021). For the 2018 harvest year, a significantly higher number of microorganisms were observed, while the 2016 harvest year

had a significantly lower number of microorganisms observed compared to the years 2015 and 2017 (Bosch et al., 2021). Bokulich et al. also reported differences between microbial communities on grapes based on harvest year with net precipitation, maximum temperature, relative humidity, latitude, and longitude most strongly influencing bacterial and fungal growth patterns as well as the taxonomic groups observed (Bokulich et al., 2014).

In addition to multiple harvest years, multiple apple-growing regions were used in our study to account for variability between regions, orchards, and packinghouse practices. However, the limited number of growers per region precluded any valid comparison between regions. Environmental conditions will differ from year to year. Based on information collected from the National Centers for Environmental Information, the Northwest region (the top apple-producing region in the United States) experienced "much below average" precipitation during harvest year 1 (2019) with "near average" temperatures, compared to year 2 (2020) when "near average" precipitation and "much above average" temperatures were reported (*2022 National Climate Report*). These year-to-year climatic changes, along with the conditions at and near the time of budding, impact both apple yield and quality.

L. monocytogenes survival varies between different apple cultivars. In our study, apple cultivar significantly impacted *L. monocytogenes* survival ($p > 0.05$) during both harvest years with greater reductions ($p > 0.05$) seen on Gala compared to Granny Smith and Honeycrisp. Macarisin et al. also observed significantly lower *L. monocytogenes* survival on unwaxed Red Delicious as compared to Fuji apples after 160 days of simulated commercial storage. Additionally, decreased survival was observed, but not always significant, on unwaxed Red Delicious apples compared to unwaxed Granny Smith apples during 160 days of storage (Macarisin et al., 2019). However, Sheng et al. reported similar survival of *L. monocytogenes*

on Granny Smith and Fuji apples during 90 days of air storage (Sheng et al., 2017). Our findings align with both Sheng et al. and Macarisin et al., with *L. monocytogenes* decreasing 1–2 log on Granny Smith apples after 30 days of refrigerated storage (Macarisin et al., 2019; Sheng et al., 2017).

Variations in surface texture and structure between apple cultivars may help explain the observed differences in *L. monocytogenes* attachment and survival. For example, Pietrysiak and Ganjyal found that Gala apples had narrower microcracks (10–100 μm), both in the stem bowl and on the equatorial surface, as compared to Granny Smith (50–150 μm) (Pietrysiak & Ganjyal, 2018). Additionally, Gala had smaller microcracks (5 μm) containing internal vertical wax platelets, whereas Granny Smith apples were covered with shallow, wider microcracks (50 μm) and crystalline wax platelets. These microcracks, as well as lenticels and trichomes on the apple surface, can reportedly serve as additional attachment sites for *Listeria innocua* and *Escherichia coli* O157:H7 (Burnett et al., 2000; Pietrysiak et al., 2019). Decreased *L. monocytogenes* survival on Gala compared to Granny Smith apples in our study reflects these reported differences in surface morphology.

Multiple studies have shown that pathogen survival in apples is partly dependent on the varying acid, sugar, and polyphenol profiles between cultivars (Nybom et al., 2020). Jelodarian et al. assessed four apple cultivars for antimicrobial activity against eleven bacterial foodborne pathogens, two cultivars of which exhibited significantly higher antimicrobial activity (Jelodarian et al., 2013). Additionally, when Alberto et al. assessed the ability of skin phenolic compounds from Royal Gala and Granny Smith to inhibit *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Enterococcus faecalis*, and *Listeria monocytogenes*, Granny Smith apples with higher phenolic content exhibited greater antimicrobial activity

against *L. monocytogenes* compared to samples with a lower phenolic content (Alberto et al., 2006). Since the antioxidant properties of apples also differ between cultivars and harvest seasons (Lata et al., 2005), the differences between the apple cultivars in our study could also be attributed to a combination of these effects.

As discussed earlier, biofilm-derived cells survived longer ($p > 0.05$) on *L. monocytogenes*- inoculated Gala and Honeycrisp apples compared to cells grown planktonically. Our findings reflect the enhanced ability of biofilm-grown cells of *L. monocytogenes* to persist longer compared to planktonically-grown cultures. The structure of a biofilm protects *L. monocytogenes* from various environmental stressors, including disinfectants and sanitizers, leading to long-term survival and persistence in such facilities (V Ferreira et al., 2014). However, the current research available is still not clear on whether genetic markers are leading to biofilm persistence (Finn et al., 2023). Once introduced into apple storage facilities and packinghouses, *L. monocytogenes* can form biofilms in difficult-to-clean locations such as drains, conveyor belts, waxing and packaging equipment, floors, foot baths, and other niches (Borucki et al., 2003; Colagiorgi et al., 2017; V Ferreira et al., 2014; Finn et al., 2023; Pietrysiak et al., 2019; Zhu et al., 2017). While there is robust information to support that *L. monocytogenes* strains are persistent in food processing environments, there is still a need to understand the mechanisms *L. monocytogenes* uses to persist in this environment (Finn et al., 2023).

3.6 Conclusions

The 2015 listeriosis outbreak traced to ice cream confirmed that even low-level contaminated products that do not support *L. monocytogenes* growth can cause life-threatening illness in highly susceptible populations (Pouillet et al., 2016). Our study shows that *L. monocytogenes* can survive on the surface of apples for at least seven months. Therefore, future risk assessments need to account for the survival of apples during long-term refrigerated storage.

Prevention of *L. monocytogenes* contamination was recently identified as the leading food safety topic of concern among apple packers (Atis et al., 2020). Recent environmental sampling for *Listeria* spp. prevalence was done in five Washington state apple packinghouses. It was shown that the food contact surfaces most likely to harbor *Listeria* spp. were polishing brushes, stainless steel dividers, brushes under fans and blowers, and dryer rollers (Ruiz-Llacsahuanga et al., 2021). Therefore, in the future, emphasis needs to be given to eradicating *L. monocytogenes* from these difficult-to-clean niches that are prone to biofilm formation.

3.7 Acknowledgments

This work was supported by the WSDA SCBGP grant #K2546 and CPS Campaign for Research. We acknowledge the contributions of Sophia Kathariou, Cameron Parsons, and Duncan Matthews in the construction of *L. monocytogenes* strains harboring specific DNA barcodes and Michael James for assistance with the apple inoculations.

CHAPTER 4: COMPOSITION AND MORPHOLOGY OF CUTICULAR WAXES IN GALA AND HONEYCRISP APPLE (*MALUS DOMESTICA*) FRUITS

Sloniker, N. and VanderWeide, J.

4.1 Abstract

The surface of an apple is made up of a naturally formed protective barrier called the cuticle. Cuticular waxes are composed of a mixture of very long-chain aliphatic compounds including fatty acids, alcohols, alkanes, aldehydes, ketones, and alkyl esters as well as cyclic compounds including triterpenoids. The objective of this study was to investigate the chemical composition and morphology of cuticular wax in apple fruit of two prominent cultivars. Apple waxes were extracted and then GC/MS analysis was performed to quantify the cuticular wax components in apple peel samples. Additionally, apple fruit were examined using a Digital Microscopy for a non-invasive examination of surface roughness. There was a significant ($p < 0.001$) difference between nonacosan-10-ol content (μg/cm^2 of peel) in the cuticular waxes for Honeycrisp (5.37±1.04) compared to Gala (0.33±0.01). Gala apples had more alkanes (g/cm2 of peel) overall for alkane compounds compared to Honeycrisp. In Gala apples the main component of the cuticular wax was nonacosane. There was significantly ($p < 0.01$) more (μg/cm^2 of peel) nonacosane in the cuticular wax of Gala (108.77±0.36) compared to Honeycrisp (46.12±4.86). The main component of the cuticular wax in Honeycrisp apples was ursolic acid. There was a significant ($p < 0.05$) difference between the oleanolic acid content (μg/cm^2 of peel) in the cuticular waxes for Honeycrisp (25.63±8.78) compared to Gala (10.80±1.67)**.** For the Gala and Honeycrisp apples, the Arithmetical Mean Height (Sa) ranged from 1.46 to 1.83 μm and 1.37 to 1.83 μm, respectively. There was no significant difference between the two cultivars (*p-value*> 0.05) for Sa.

4.2 Introduction

Apples (*Malus domestica*) are produced by the apple tree and are part of the plant family *Rosaceae*, sub-family *amygdaloideae*. This category of fruit has a center "core" of about two to five small seeds encased in carpels and surrounded by edible flesh. Other common fruits in this category include pear and quince fruit (Musacchi, 2018). Cultivated apples are thought to have been domesticated over 4,000 years ago in Central Asia from a wild apple *Malus sieversii* (Britannica, 2023). The wild apple then spread to western Europe and hybridized with other wild apples and before settlement to the Americas, hundreds of distinct apple cultivars were recognized in Europe (Britannica, 2023).

China is currently the number one producer of apples, with the United States coming in second followed by Turkey (Britannica, 2023). For growing seasons 2018-2022, the average apple crop in the United States was 258 million bushels (US Apple, 2023). According to the USDA Economic Research Service, apples were the most consumed fruit in the United States for 2019 (U. S. Department of Agriculture Economic Research Service, 2021). Additionally, of the seven most consumed fruits, apples were the only fruit available in five different consumption forms fresh, canned, frozen, dried, and juice (U. S. Department of Agriculture Economic Research Service, 2021).

There are three different classes of apples cider, cooking, and dessert (Britannica, 2023). Cultivars in the dessert category prioritize color, size, aroma, smoothness, and flavor. Dessert cultivars that are popular in the United States include Honeycrisp, Gala, Red Delicious, Golden Delicious, and Granny Smith (Britannica, 2023). In the United States, Gala apples are the most produced apple cultivar accounting for roughly 18% of the market, followed by Red Delicious, and Honeycrisp for the top three (US Apple, 2023). In this

paper, we analyzed Gala and Honeycrisp apples. The Gala cultivar originated in New Zealand and is a cross between Kidd's Orange Red and Golden Delicious. This cultivar was brought to the United States in the early 1970s (US Apple, 2023). The Honeycrisp apple was developed by the University of Minnesota and is a cross between Keepsake and MN1627, and an unknown Minnesota line (US Apple, 2023). The surface of an apple is made up of a naturally formed protective layer called the cuticle.

The cuticle is on the outer surface of the epidermal cell wall of aerial plant organs such as stems, leaves, flowers, and fruits (Leide, 2018; Serrano et al., 2014). The cuticle is a barrier that protects the fruit by preventing water loss, shielding against mechanical damage and harmful irradiation, as well as providing a line of defense against insects, fungi, and pathogenic microorganisms (Dominguez et al., 2017; Serrano et al., 2014). The structure of the cuticle consists of two main components, the outermost cuticle proper and the cuticular layer which sits between the cuticle proper and the cell wall. Throughout the cuticle proper and the cuticular layer is a cutin matrix that is interspersed with and covered by wax. On the outermost layer of the cuticle proper are epicuticular wax crystals.

Cuticular waxes are composed of a mixture of very long-chain aliphatic compounds including fatty acids, alcohols, alkanes, aldehydes, ketones, and alkyl esters as well as cyclic compounds including triterpenoids. In apples, cuticular waxes consist of primarily alkanes (50-80%), fatty acids (10-30%), and alcohols (1-20%) (Chai et al., 2020). Regarding alkanes, the compounds most often observed in the highest concentration include nonacosane (C29) and heptacosane (C27) (Chai et al., 2020; Chen, 2021; Klein, 2020; Wang et al., 2022). For fatty acids the compounds most often observed in the highest concentration include tetradecanoic acid (C14), hexadecenoic acid (C16), and octadecanoic acid (C18) (Chai et al., 2020; Chen, 2021;

Klein, 2020; Wang et al., 2022). Moreover, for alcohols, the compounds most often observed in the highest concentration include tetracosanol (C24) and nonacosan-10-ol (C29) (Chai et al., 2020; Chen, 2021; Klein, 2020; Wang et al., 2022). The aldehyde compound most commonly observed in the highest concentration was octacosanal and for triterpenoids, ursolic acid and oleanolic acid were both consistently reported as being present in the cuticular waxes (Klein, 2020). Wax composition is influenced by apple cultivars and storage time. Both alkanes and primary alcohols were found to decrease during storage while fatty acids increased during storage time (Chai et al., 2020).

The cuticular wax composition has been associated with important postharvest quality parameters including water loss, and physical and/or biological stress (Riederer & Schreiber, 2001; Singh et al., 2018). Additionally, some studies have been done on the ability of the cuticular wax composition to impact food safety. In 2020, a study found attachment and removal of *E. coli* K12 on five leafy green cultivars was influenced by wax composition, bacterial removal was positively correlated with alkanes, ketones, and total wax content on the surface of the leaf. Samples with higher wax content had less surface roughness and higher reported bacterial removal compared to a lower wax content. Also, higher reported surface roughness increased bacterial adhesion and decreased bacterial removal (Palma-Salgado et al., 2020). A study in 2018 also found the structure of the cuticular wax influenced bacterial attachment as *Listeria innocua* was found to adhere more readily to gaps between waxes and not on smooth surfaces (Pietrysiak & Ganjyal, 2018).

The influence of cuticular wax composition and structure is important to investigate as it impacts apple fruit quality and food safety. The objective of this study was to investigate the

chemical composition and morphology of cuticular wax in apple fruit of two prominent cultivars.

4.3 Materials and methods

Apple samples. Gala and Honeycrisp apples were grown in an orchard in Sparta, MI, and were harvested during fall 2023. The apples were directly transported to Michigan State University, East Lansing, MI on September 1, 2023, and were stored at 4 °C for five days until analysis. Apples were washed with DI water and air dried before extraction.

Chemicals and standards. HPLC grade chloroform, pyridine, and BFSTA+TMCS (99:1) used for wax extraction and derivatization were purchased from Sigma Aldrich, Missouri, USA. Internal standard tetracosane and authentic standards heptacosane, tetracosanol, myristic acid, and aleanolic acid were purchased from Sigma Aldrich, Missouri, USA. Additional authentic standards hexadecanal and trans-β-farnesene were purchased from Cayman Chemical, Michigan, USA.

Extraction of cuticular waxes. Cuticular waxes were extracted according to Yan, et al., with some modifications (Yan et al., 2023). For each sample, three apples were used and from each apple five rectangles (10x20 mm each) were cut from the equator section with a blade. The 15 skin samples were submerged in a 50 mL Erlenmeyer flask with 20 mL of chloroform for 60 s with gentle swirling. A single chloroform bath was performed for each replicate with the addition of 10 μg of tetracosane as the internal standard. After 60 s, 5 mL of chloroform was added to the Erlenmeyer flask to wash off any waxes attached to the wall of the flask. The extractants were mixed well and transferred to 15 mL testing tubes for drying under a nitrogen evaporator (Techtongda). The dried samples were stored at − 20 °C until further analysis.

Derivatization of cuticular waxes. Dried waxes were resuspended into 500 μL of chloroform and transferred into a 2 mL amber vial (Agilent Technologies Inc., California, USA) before drying under a nitrogen stream to total dryness. For the silylation of the hydroxyl

and carboxylic acid groups, reagents BFSTA + TMCS (99:1) and pyridine were added to the vials, and the vials were held at 80 °C for 1 h in a water bath (ThermoFisher Scientific, Waltham, MA, USA). After 1 h the samples were again dried under the nitrogen stream and reconstituted with 500 μL. Then the samples were filtered using a 0.22 μm PVDF Millex filter (VWR, Pennsylvania, USA).

GC-MS analysis and quantification of cuticular waxes. GC/MS analysis was performed to quantify the cuticular wax components in apple peel samples using an Agilent 7890A GC/ single quadrupole mass spectrometer with 5975C inert XL MSD (Agilent, Santa Clara, CA). One μL of the derivatized sample was injected in a split mode (1:100) with an injector temperature of 275°C and a flow rate of 1.0 mL/min helium. The syringe pre- and post-inject delay was set to 3000 ms. Separation was achieved on an Agilent J&W VF5ms column (30 m x 0.25 mm x 0.25 μm) (Agilent, Santa Clara, CA) using the following temperature profile: 40°C for 1 min; 40°C min^{-1} to 275°C; 10°C min^{-1} to 325°C; 325°C for 10 min. Ionization employed 70 eV electron ionization and the mass spectrometer was operated in scanning mode with a scan range of m/z 50 to 600. Cuticular wax components were identified based on background-subtracted spectrum matches to the NIST17 library. A quantifier and qualifier ion were used to quantify the peak areas for each compound. Tetracosanol, hexadecanal, heptacosane, α-farnesene, tetradecanoic acid, and oleanolic acid were used as representative standards for alcohols, aldehydes, alkanes, alkenes, fatty acids, and triterpenoids, respectively. The content of cuticular waxes of each sample was expressed in terms of wax (μg) per surface area (cm^2) extracted.

Roughness measurements. Fresh, whole apples were viewed using Digital Microscopy for a non-invasive examination of surface roughness. A Keyence VHX-6000 (Keyence

Corporation, Osaka, Japan) microscope was used at the Michigan State University Center for Advanced Microscopy. The roughness of the apple surface was measured using a 3D Surface Profiler (number of pixels 1920 [H]x1080 [V]). Five tile stacked images of a 400x1000 μm rectangle were taken for each apple at a magnification of 1000x. For each image, Arithmetical Mean Height (Sa) (μm) and Maximum height (Sz) (μm) were recorded (Keyence Corporation, 2023). The Sa is an absolute value that expresses the difference in height of each point compared to the arithmetical mean of the surface. The Sz is the sum of the maximum peak height value (Sp) (μm) and the maximum pit height value (Sv) (μm) (Keyence Corporation, 2023).

Statistical analysis. Four biological replicates with three apples each were analyzed for wax composition. All cuticular wax data is expressed as means ± standard error. Each of the four biological replicates also had two technical replicates. For surface imaging, four apples were analyzed at five 400x1000 μm sections along the equator of the apple. All data are presented as mean values with their standard errors. Students t-tests were used to determine statistical significance between the cultivars. All statistical analysis was conducted in RStudio Version 2023.06.1+524 (RStudio, Inc., Boston, MA, USA).

4.4 Results

Alcohols. More total alcohols were detected in the cuticular wax of Honeycrisp apples compared to Gala (Figure 5) (Table 3). There was no significant ($p > 0.05$) difference between Gala and Honeycrisp apples for alcohols (μg/cm^2 of peel) 1-hexacosanol, 1-octacosanol, 1-tetracosanol, and 1-triacontanol. There was a significant ($p < 0.001$) difference between nonacosan-10-ol content (μg/cm^2 of peel) in the cuticular waxes for Honeycrisp (5.37±1.04) compared to Gala (0.33±0.01).

Aldehydes. For the two aldehyde compounds analyzed, there was no significant ($p > 0.05$) difference in triacontanal (μg/cm^2 of peel) between Gala (5.65±0.79) and Honeycrisp (6.17±0.77) (Figure 6) (Table 3). For both Gala and Honeycrisp apples no (< LOD) hexadecanal was detected (μg/cm^2 of peel).

Alkanes. Gala apples had more alkanes (g/cm2 of peel) overall for alkane compounds compared to Honeycrisp (Figure 7) (Table 3). There was no significant ($p > 0.05$) difference between Gala and Honeycrisp apples for the alkane (μg/cm^2 of peel) heptacosane. In Gala apples the main component of the cuticular wax was nonacosane. There was significantly ($p < 0.01$) more (μg/cm^2 of peel) nonacosane in the cuticular wax of Gala (108.77±0.36) compared to Honeycrisp (46.12±4.86).

Alkenes. For alkene compounds, there was significantly ($p < 0.001$) more α-farnesene (μg/cm^2 of peel) in Honeycrisp (1.04±0.06) apples compared to Gala (< LOD) (Figure 8) (Table 3).

Fatty acids. Among the six fatty acids analyzed, there was no significant ($p > 0.05$) difference between Gala and Honeycrisp apples for fatty acids (μg/cm^2 of peel) hexacosanoic acid, octacosanoic acid, and palmitic acid. For both Gala and Honeycrisp apples, no (<

LOD) stearic acid or tetradecanoic acid was detected (μg/cm^2 of peel) (Figure 9) (Table 3). There was a significant ($p < 0.05$) difference between the triacontanoic acid content (μg/cm^2 of peel) in the cuticular waxes for Honeycrisp (2.95±0.06) compared to Gala (2.79±0.02).

Triterpenoids. There was no significant ($p > 0.05$) difference between Gala and Honeycrisp apples for triterpenoids (μg/cm^2 of peel) ursolic acid and uvaol (Figure 10) (Table 3). The main component of the cuticular wax in Honeycrisp apples was ursolic acid. There was a significant ($p < 0.05$) difference between the oleanolic acid content (μg/cm^2 of peel) in the cuticular waxes for Honeycrisp (25.63±8.78) compared to Gala (10.80±1.67).

Arithmetical Mean Height (Sa) and Maximum height (Sz). For the Gala and Honeycrisp apples, the Arithmetical Mean Height (Sa) ranged from 1.46 to 1.83 μm and 1.37 to 1.83 μm, respectively (Table 4). There was no significant difference between the two cultivars (p-value> 0.05) for Sa. For the Gala and Honeycrisp apples, the Maximum height (Sz) ranged from 12.26 to23.99 μm and 11.97 to 16.37 μm, respectively (Table 4). There was no significant difference between the two cultivars (p value > 0.05) for Sz.

Table 4. Sa and Sz means ± SE for Gala and Honeycrisp apple samples

	Gala	**Honeycrisp**	**Gala**	**Honeycrisp**
	Sa (μm)	**Sa (μm)**	**Sz (μm)**	**Sz (μm)**
Sample 1	1.83±0.53	1.72±0.40	13.30±2.50	12.83±1.77
Sample 2	1.46±0.46	1.75±0.62	12.26±2.01	16.37±7.07
Sample 3	1.70±0.86	1.37±0.26	23.99±13.91	11.97±4.90
Sample 4	1.68±0.58	1.83±0.87	14.73±5.75	15.63±3.58
Average	1.67	1.67	16.07	14.20
p value	0.99		0.40	

4.5 Discussion

In a study on the components of cuticular waxes in apples, authors compared the cultivars Golden Delicious and Granny Smith. Similar results were found between the two cultivars for the alcohol portion of the waxes (Fernandez-Moreno et al., 2016). However, high concentrations of nonacosan-10-ol were detected for Granny Smith apples, while little was detected in Golden Delicious apples. Similar results were reported in our research where Honeycrisp apples had a significantly higher amount of the alcohols compared to Gala apples. Nonacosan-10-ol is one of the primary components that make up the crystalline structure of the epicuticular waxes (Yang et al., 2017). The quality parameter of greasiness in Jonagold and Elstar apples is impacted by the nonacosan-10-ol content in the cuticular waxes, however, Gala apples still exhibit greasiness during ripening without much nonacosan-10-ol in the wax layer (Yang et al., 2017).

Alkanes heptacosane and nonacosane were analyzed in this study. Alkanes were the only compound group where Gala apples had more of each compound than Honeycrisp apples. Alkanes were the most abundant compound class for Gala apples and the second most abundant for Honeycrisp apples. Nonacosane was the compound with the highest amount for Gala apples. Other studies also align with nonacosane as a major alkane present in the cuticular waxes of apple fruit (Belding et al., 2000; Dong, 2012; Leide, 2018; Morice, 1973). Nonacosane is also the most abundant alkane in the cuticular waxes of blueberries, plums, and sweet cherries (Lara, 2014). Nonacosane is a straight-chain alkane with a biological role in plants as a metabolite and a volatile oil component. In a study reporting good cytotoxic and antibacterial activity of the volatile oil component of apples, researchers found nonacosane was the primary compound present. They contributed the high amount of nonacosane present

to the cytotoxic activity against human lung carcinoma cells as well as a high antibacterial activity against *Aspergillus flavus*, *Staphylococcus aureus*, and *Escherichia coli* (El-Hawary, 2018).

The alkene alpha-farnesene was present in the cuticular wax of Honeycrisp apples and was below the limit of detection in Gala apples. Previous research supports the lower levels observed of alpha-farnesene observed in Gala apples (Souleyre et al., 2019). This research also showed that alpha-farnesene exhibited lower infection of post-harvest pathogens *Colletotrichum acutatum*, *Penicillium expansum*, and *Neofabraea alba* (Souleyre et al., 2019). Alpha-farnesene is also present in pear, and quince coatings, both fruits that are part of the plant family *Rosaceae*, sub- family *amygdaloideae* with apples (Huelin & Murray, 1966).

Regarding the fatty acid content observed in the cuticular wax layer, we reported similar concentrations across hexacosanoic acid, octacosanoic acid, palmitic acid, and triacontanoic acid. Other research has shown a higher concentration of triacontanoic acid compared to the other fatty acids present in the cuticular wax layer (Fernandez-Moreno et al., 2016; Leide, 2018). However, similar results to ours were seen in Golden Delicious and Red Delicious apples for hexacosanoic acid, octacosanoic acid (Wang et al., 2022).

Triterpenoids were the most abundant compound class for Honeycrisp apples. Ursolic acid was the compound with the highest amount for Honeycrisp apples, this is consistent with other research showing ursolic acid typically composes between 30 – 72% of total cuticular waxes (He et al., 2014; Leide, 2018). Ursolic acid is primarily found in the peel of berries and its biological role in plants is to protect against pathogens. Second to ursolic acid was oleanolic acid, both are pentacyclic triterpenoids that are common in plants (Jesus et al., 2015). While oleanolic acid has been found in over 2000 plants, it is most common in the

Oleaceae (olive) family. The biological role most associated with oleanolic acid is to create a barrier to protect the plant from water loss and pathogens. Both ursolic and oleanolic acid have been shown to have antimicrobial properties against human pathogens as well including methicillin-resistant *Staphylococcus aureus*, *Bacillus cereus,* and *Enterococcus faecalis* (Jesus et al., 2015).

The surface roughness of fresh produce has been studied concerning the impact on food safety. Specifically, the correlation between the roughness of the food surface and the attachment of bacteria or fungi to the surface (Wang et al., 2009). For fruits that have been measured for surface roughness, apples have frequently been reported to have the least rough surface compared to oranges, cantaloupes, and avocados (Bhide et al., 2017). The difference in surface roughness between fruit cultivars has not previously been studied and our research shows no differences between the two cultivars. Research on the attachment of *Listeria innocua* was done on the apple peel and found bacteria attached more readily to the microcracks, lenticels, and wax platelets in the stem bowl and calyx portions of the apple and not present on the smoother equatorial portions of the apple (Pietrysiak & Ganjyal, 2018). Further research comparing the differences in surface roughness on the equatorial section, stem bowl, and calyx portions would be valuable.

4.6 Acknowledgements

We would like to acknowledge the contributions of both the Mass Spectrometry and Metabolomics Core and the Center for Advanced Microscopy for the GC-MS analysis and surface roughness imaging as well as Dr. Randy Beaudry for providing the apple fruit.

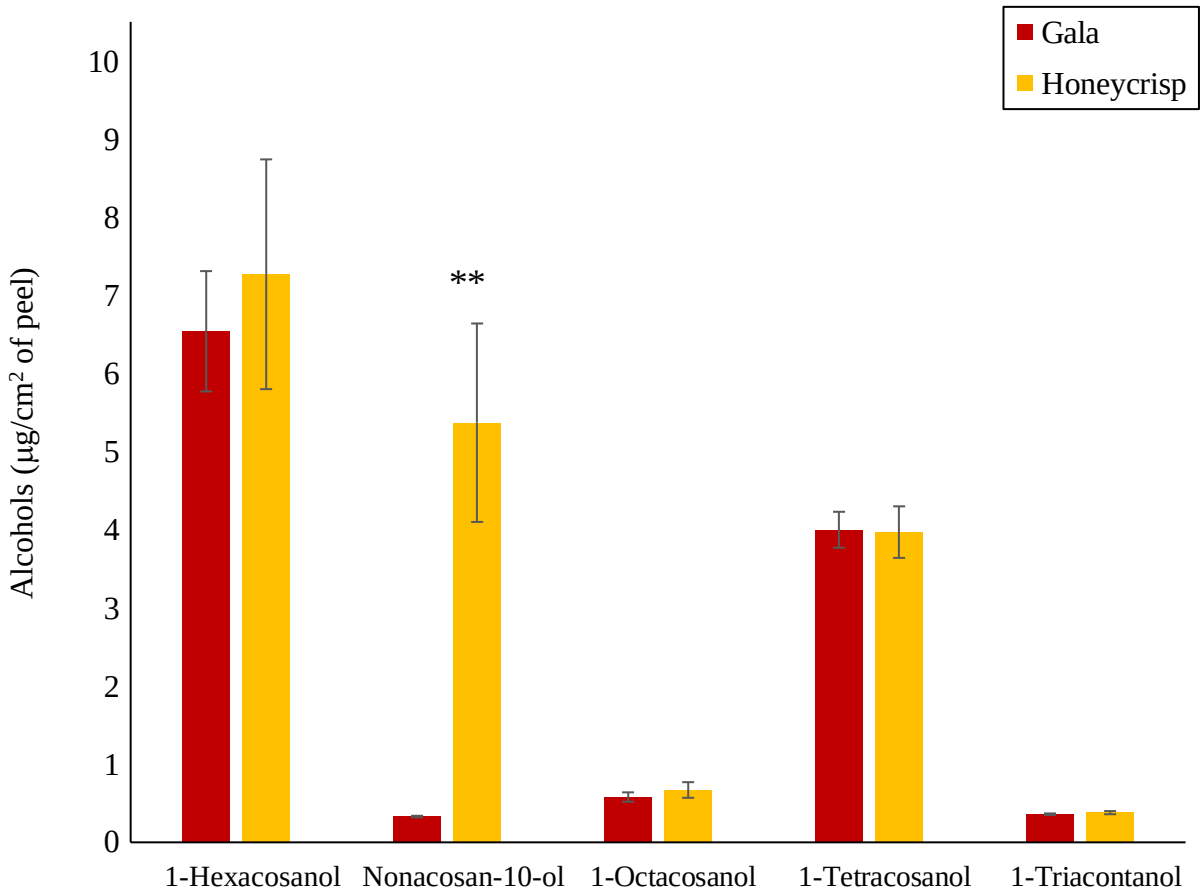

Figure 5. Cuticular wax composition for alcohol compounds (µg/cm^2 of peel) in Gala (red) and Honeycrisp (yellow) apples. The asterisk (**) is used to represent a statistically significant result ($p < 0.01$). A # indicates that the composition was not detected (< LOD).

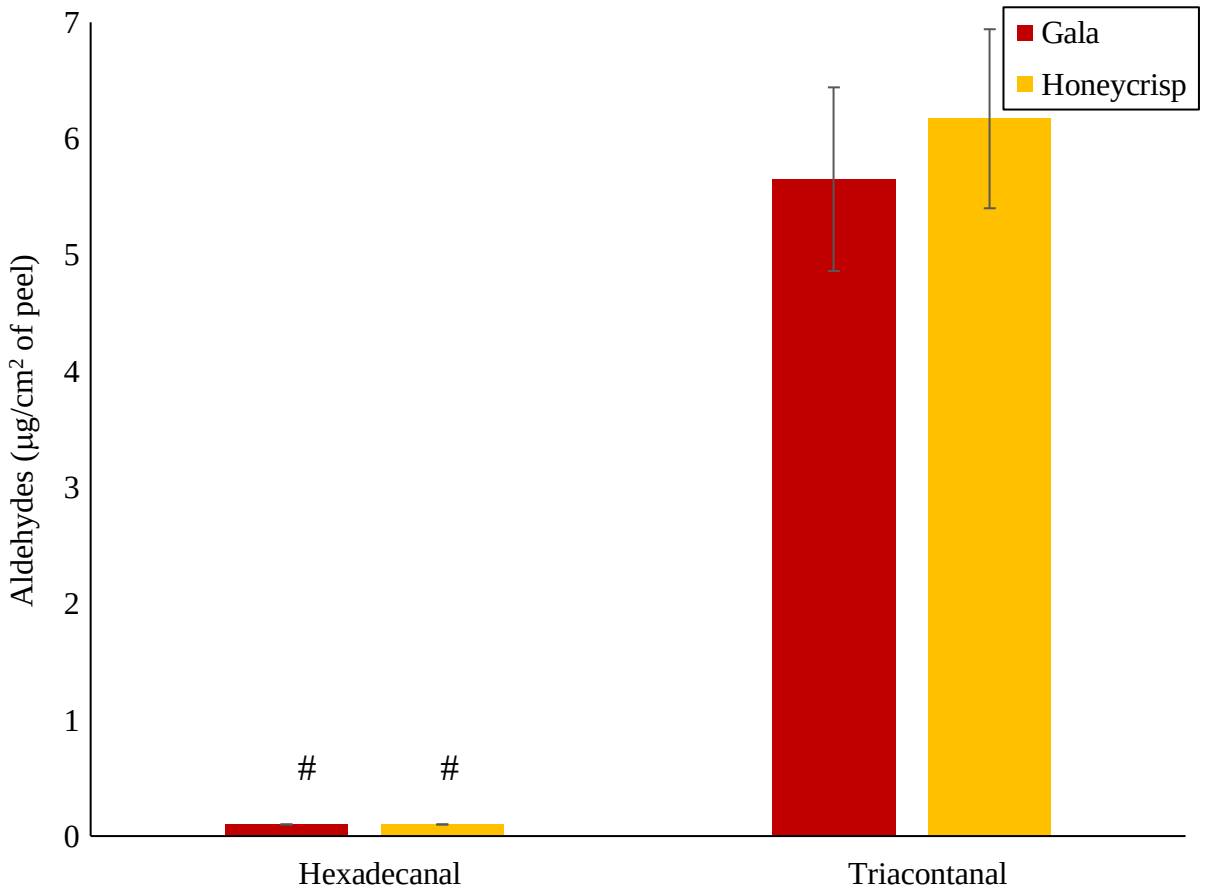

Figure 6. Cuticular wax composition for aldehyde compounds (µg/cm^2 of peel) in Gala (red) and Honeycrisp (yellow) apples. A # indicates that the composition was not detected (< LOD).

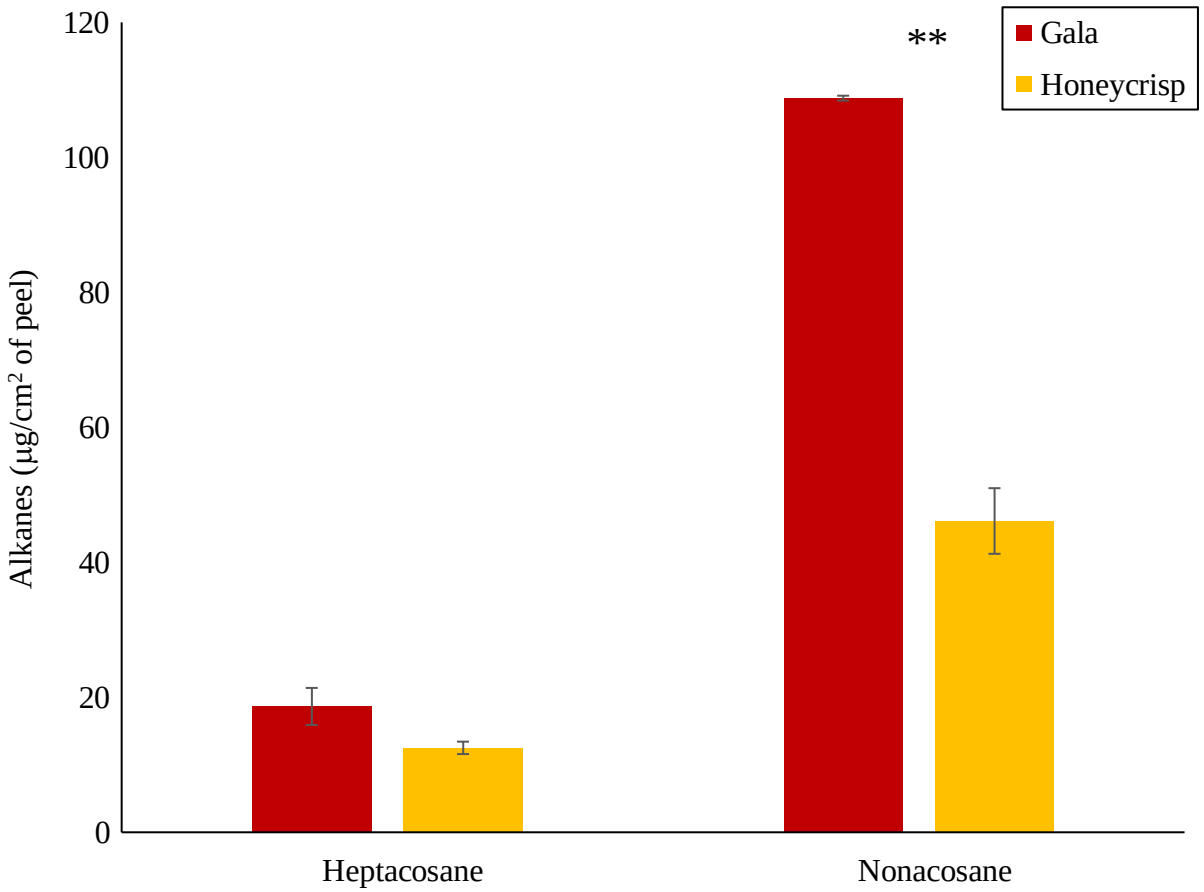

Figure 7. Cuticular wax composition for alkane compounds (µg/cm^2 of peel) in Gala (red) and Honeycrisp (yellow) apples. The asterisk (*) is used to represent a statistically significant result (p< 0.01). A # indicates that the composition was not detected (< LOD).

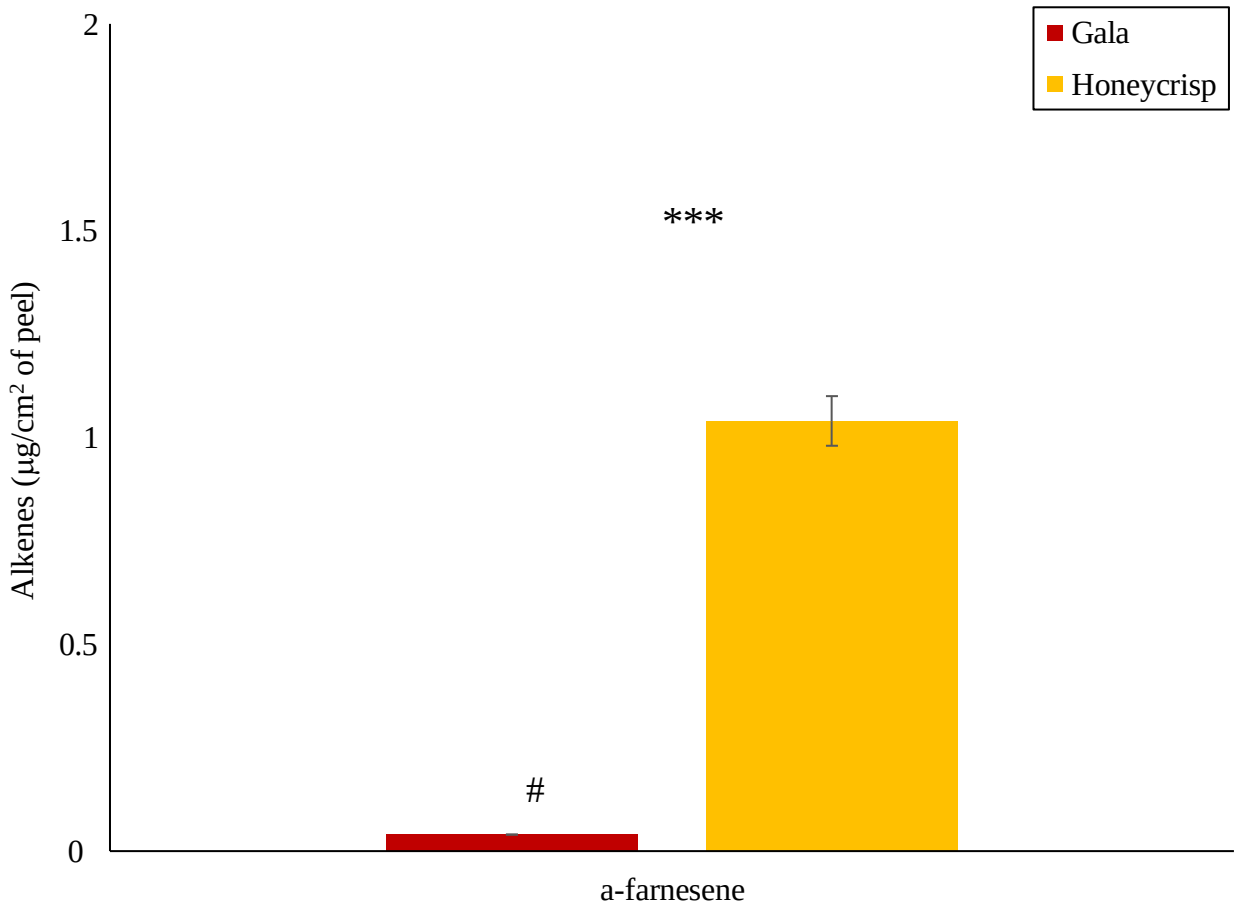

Figure 8. Cuticular wax composition for alkene compounds (µg/cm^2 of peel) in Gala (red) and Honeycrisp (yellow) apples. The asterisk (***) is used to represent a statistically significant result ($p < 0.001$). A # indicates that the composition was not detected (< LOD).

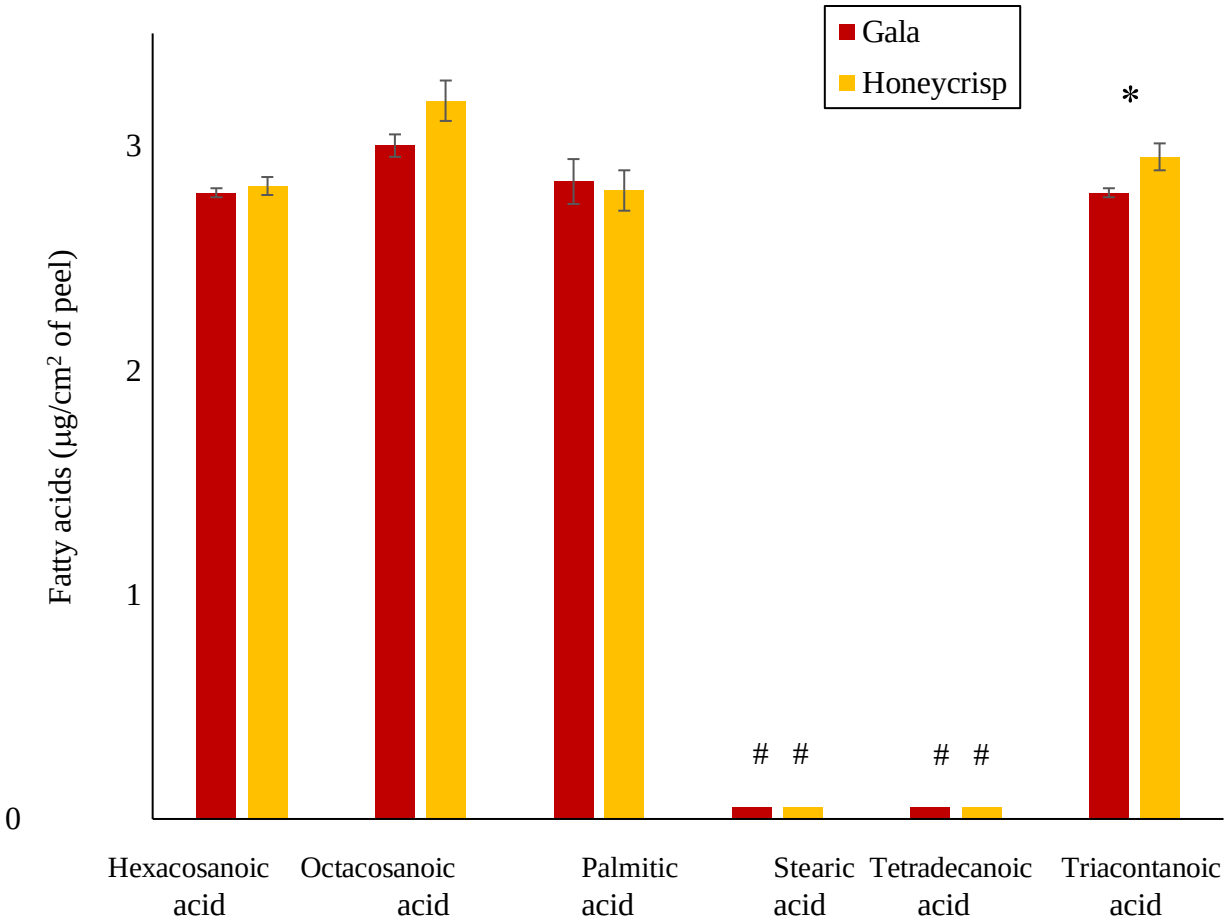

Figure 9. Cuticular wax composition for fatty acid compounds (µg/cm^2 of peel) in Gala (red) and Honeycrisp (yellow) apples. The asterisk (*) is used to represent a statistically significant result (p< 0.05). A # indicates that the composition was not detected (< LOD).

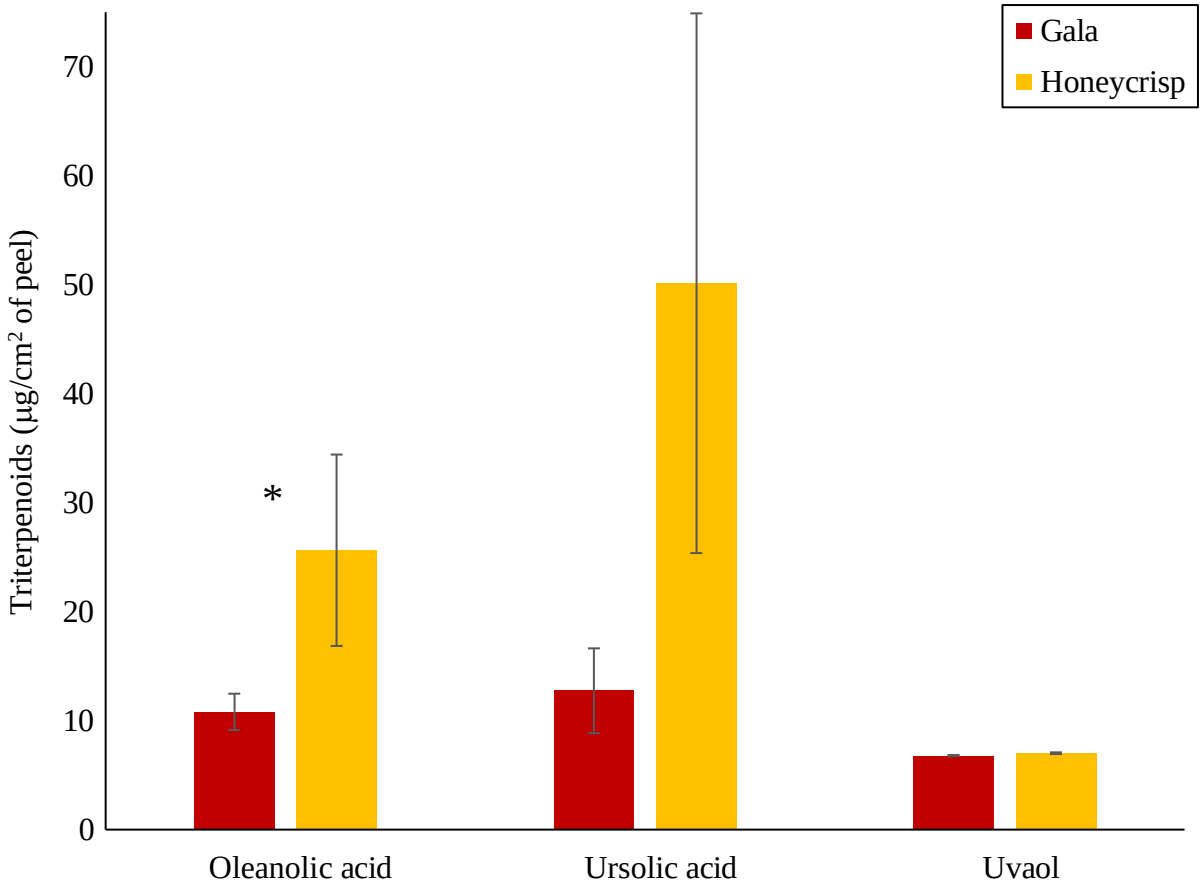

Figure 10. Cuticular wax composition for triterpenoid compounds (µg/cm^2 of peel) in Gala (red) and Honeycrisp (yellow) apples. The asterisk (**) is used to represent a statistically significant result ($p < 0.01$).

Table 3. Cuticular wax composition for Gala and Honeycrisp apples presented as means ± SE. NS represents no significant difference between the apple cultivars

Compound	**Gala ($\mu g/cm^2$ of peel)**	**Honeycrisp ($\mu g/cm^2$ of peel)**	***p* value**
Alcohols			
1-Hexacosanol	6.54±0.77	7.27±1.47	NS
Nonacosan-10-ol	0.33±0.01	5.37±1.04	< 0.01
1-Octacosanol	0.58±0.06	0.67±0.10	NS
1-Tetracosanol	4.00±0.23	3.97±0.33	NS
1-Triacontanol	0.36±0.01	0.38±0.02	NS
Aldehydes			
Hexadecanal	< LOD	< LOD	NS
Triacontanal	5.65±0.79	6.17±0.77	NS
Alkanes			
Heptacosane	18.65±2.74	12.51±0.92	NS
Nonacosane	108.77±0.36	46.12±4.86	< 0.01
Alkenes			
a-farnesene	< LOD	1.04±0.06	< 0.001
Fatty acids			
Hexacosanoic acid	2.79±0.02	2.82±0.04	NS
Octacosanoic acid	3.00±0.05	3.20±0.09	NS
Palmitic Acid	2.84±0.10	2.80±0.09	NS
Stearic acid	< LOD	< LOD	NS
Tetradecanoic acid	< LOD	< LOD	NS
Triacontanoic acid	2.79±0.02	2.95±0.06	< 0.05
Triterpenoids			
Oleanolic acid	10.80±1.67	25.63±8.78	< 0.01
Ursolic acid	12.74±3.89	50.13±24.76	NS
Uvaol	6.79±0.05	7.01±0.08	NS

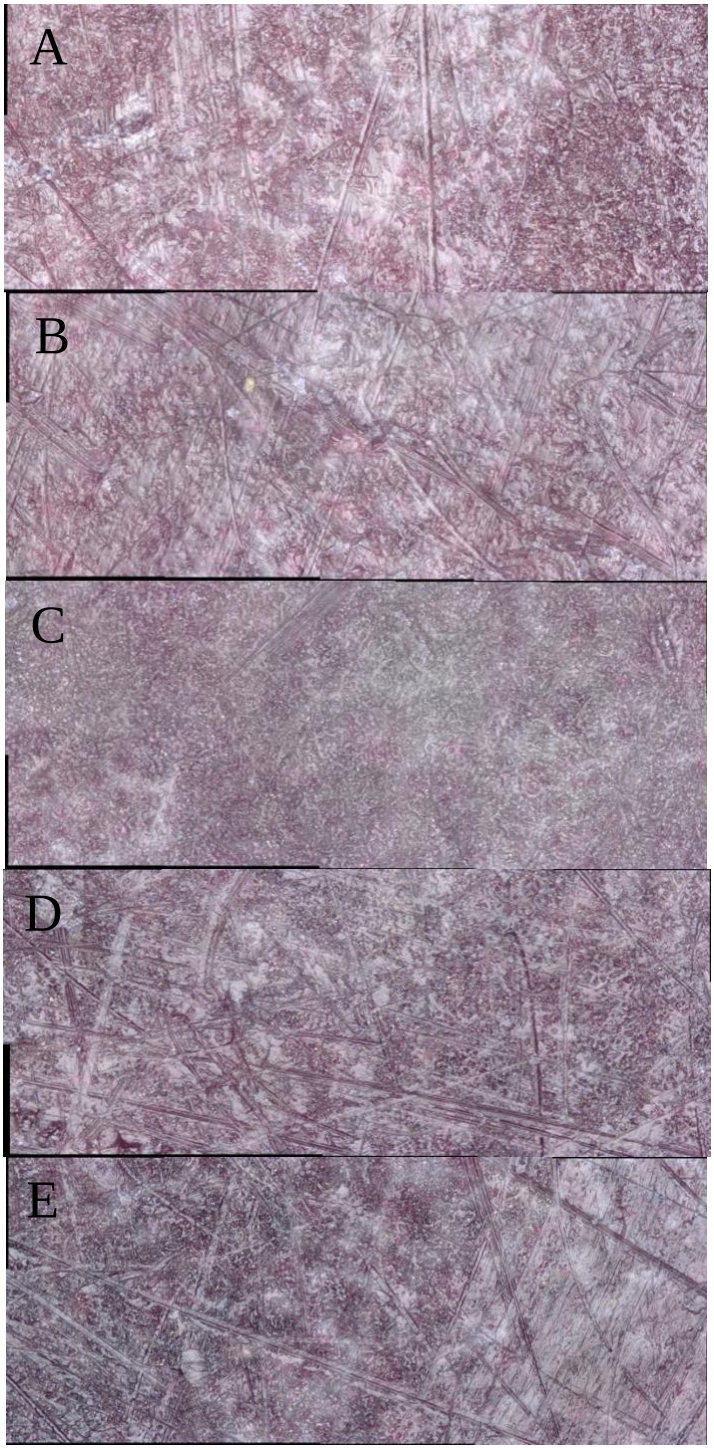

Figure 11. Gala sample 1 surface image taken with Keyence Digital Microscope VHX-6000.
(A) Gala sample 1 site 1, Sa 1.099 μm, Sz 15.216 μm, size 400.00 x 1011.032 x 15.388 μm
(B) Gala sample 1 site 2, Sa 1.829 μm, Sz 13.658 μm, size 400.00 x 1019.319 x 14.062 μm
(C) Gala sample 1 site 3, Sa 2.145 μm, Sz 13.947 μm, 400.00 x 1007.717 x 14.475 μm
(D) Gala sample 1 site 4, Sa 2.489 μm, Sz 14.806 μm, 400.00 x 1005.231 x 14.805 μm
(E) Gala sample 1 site 5, Sa 1.593 μm, Sz 8.991 μm, 400.00 x 1002.745 x 9.249 μm.

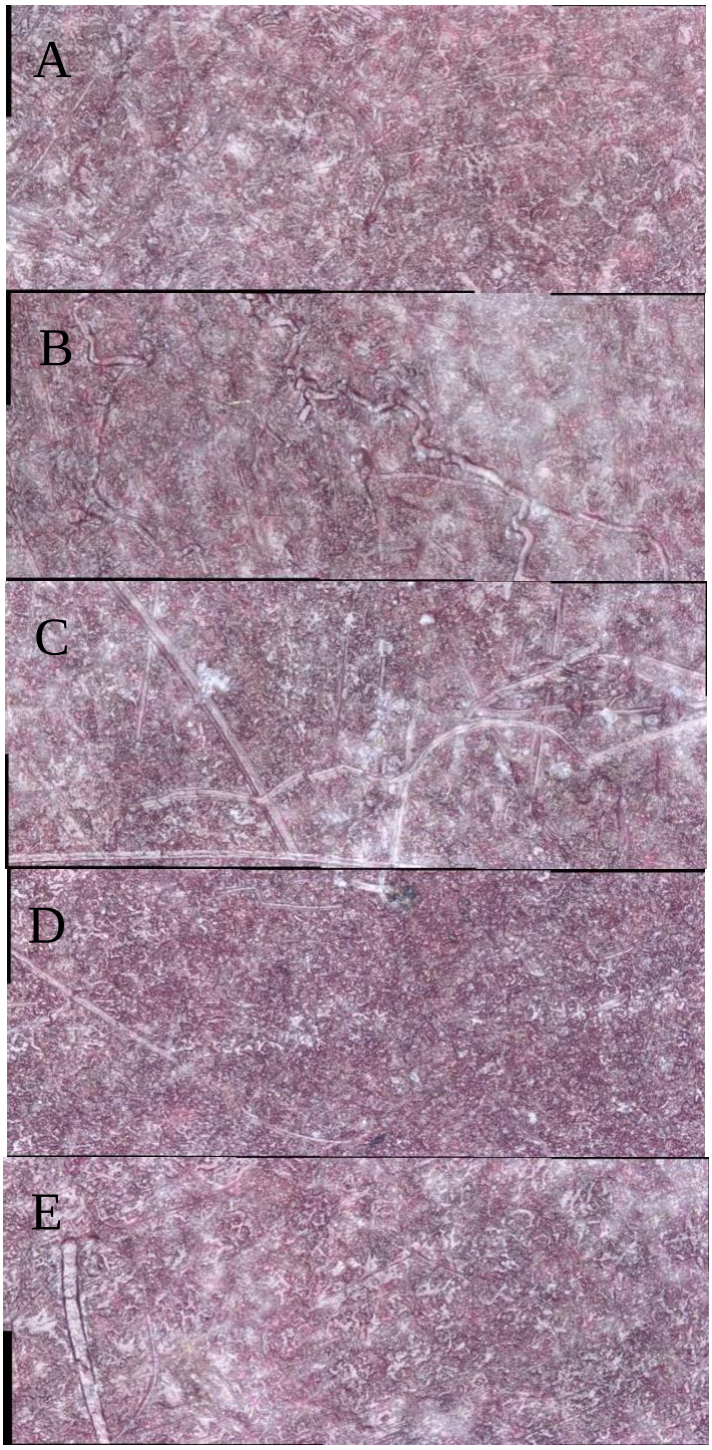

Figure 12. Gala sample 2 surface image taken with Keyence Digital Microscope VHX-6000.
(A) Gala sample 2 site 1, Sa 1.248 μm, Sz 9.658 μm, 400.00 x 1003.574 x 9.658 μm
(B) Gala sample 2 site 2, Sa 1.713 μm, Sz 11.575 μm, 400.00 x 1018.491 x 12.312 μm
(C) Gala sample 2 site 3, Sa 0.963 μm, Sz 12.249 μm, 400.00 x 1006.889 x 12.249 μm
(D) Gala sample 2 site 4, Sa 1.263 μm, Sz 12.566 μm, 400.00 x 1005.231 x 12.918 μm
(E) Gala sample 2 site 5, Sa 2.122 μm, Sz 15.227 μm, 400.00 x 1012.690 x 17.488 μm.

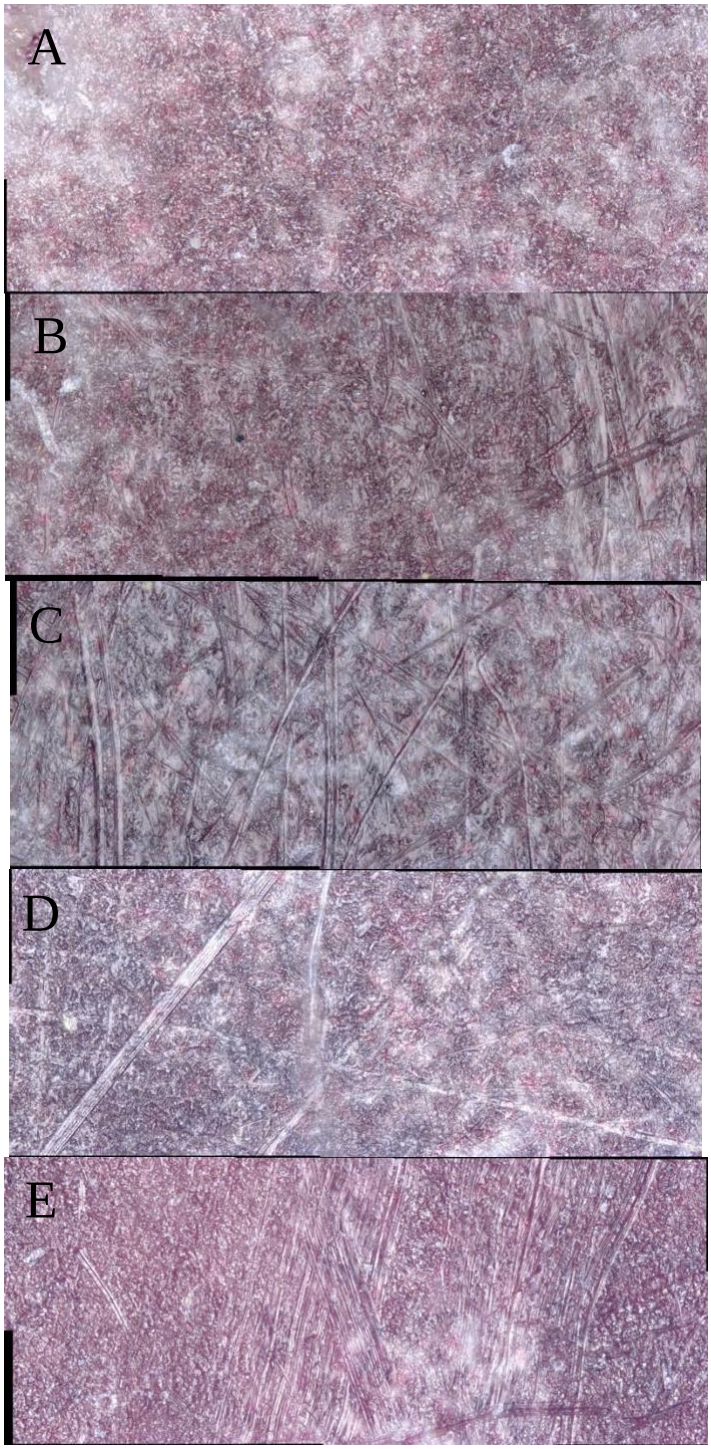

Figure 13. Gala sample 3 surface image taken with Keyence Digital Microscope VHX-6000.
(A) Gala sample 3 site 1, Sa 1.974 μm, Sz 34.778 μm, 400.00 x 1004.403 x 34.777 μm
(B) Gala sample 3 site 2, Sa 3.069 μm, Sz 18.285 μm, 400.00 x 1011.032 x 19.812 μm
(C) Gala sample 3 site 3, Sa 1.468 μm, Sz 15.724 μm, 400.00 x 1016.833 x 15.723 μm
(D) Gala sample 3 site 4, Sa 0.942 μm, Sz 42.202 μm, 400.00 x 1001.916 x 42.202 μm
(E) Gala sample 3 site 5, Sa 1.071 μm, Sz 8.982 μm, 400.00 x 1061.584 x 10.218 μm.

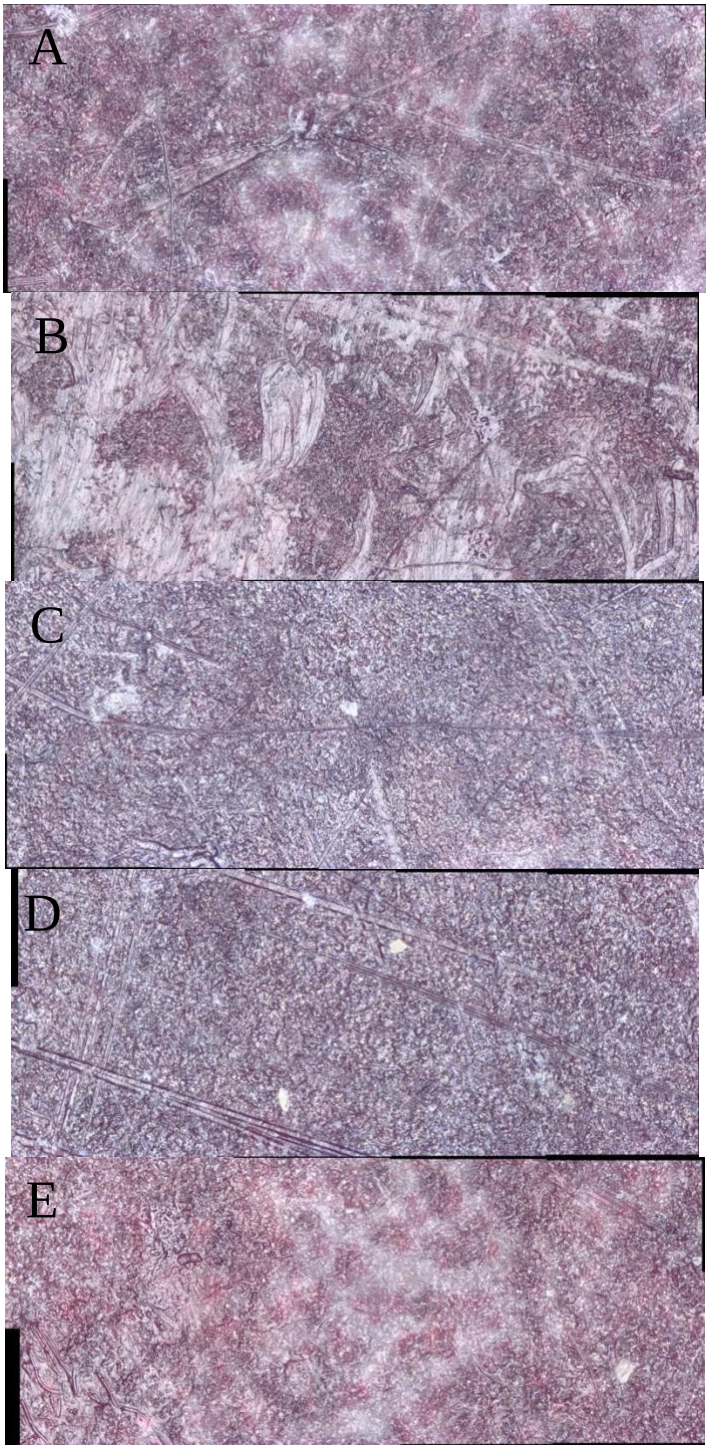

Figure 14. Gala sample 4 surface image taken with Keyence Digital Microscope VHX-6000.
(A) Gala sample 4 site 1, Sa 1.206 μm, Sz 11.460 μm, 400.00 x 1014.347 x 11.481 μm
(B) Gala sample 4 site 2, Sa 2.321 μm, Sz 15.145 μm, 400.00 x 1008.546 x 15.837 μm
(C) Gala sample 4 site 3, Sa 1.190 μm, Sz 8.694 μm, 400.00 x 1013.518 x 9.454 μm
(D) Gala sample 4 site 4, Sa 2.281 μm, Sz 14.402 μm, 400.00 x 1027.607 x 15.174 μm
(E) Gala sample 4 site 5, Sa 1.382 μm, Sz 23.837 μm, 400.00 x 1026.778 x 23.936 μm.

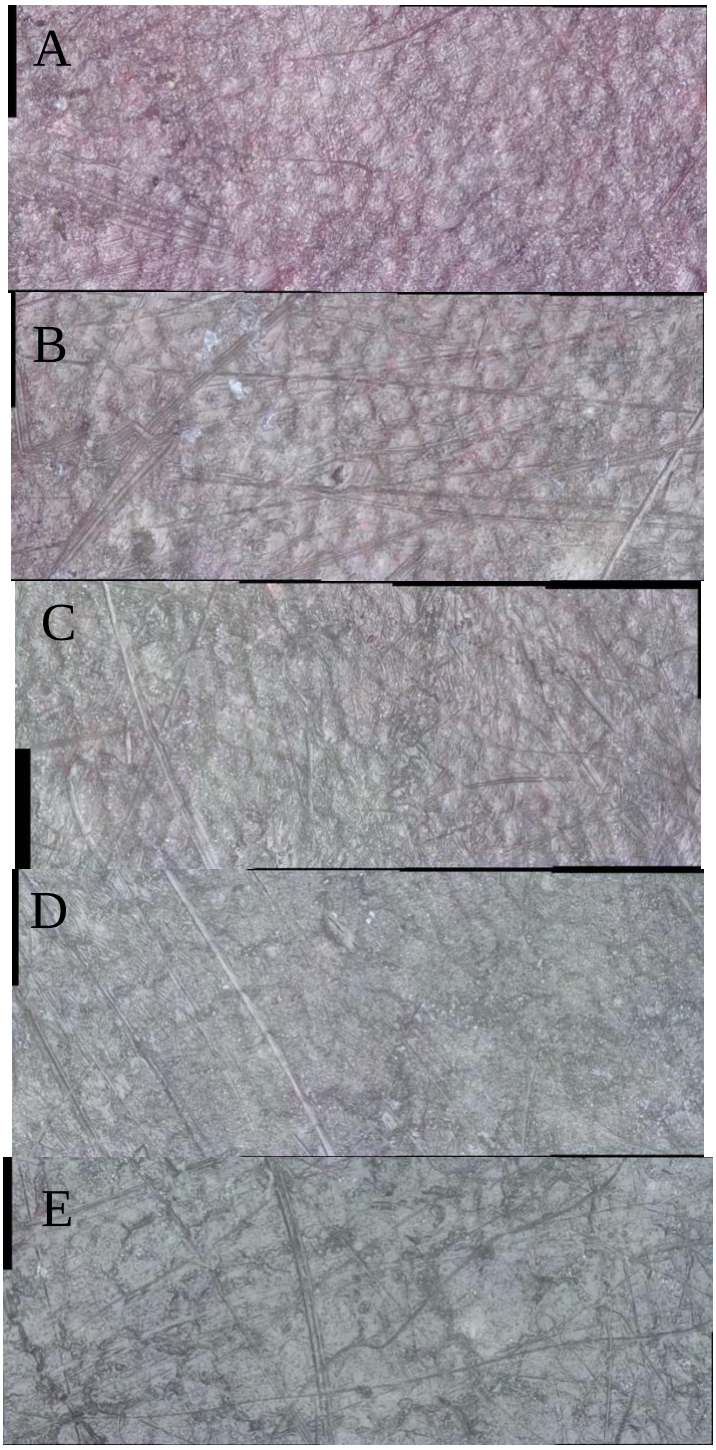

Figure 15. Honeycrisp sample 1 surface image taken with Keyence Digital Microscope VHX-6000.
(A) Honeycrisp sample 1 site 1, Sa 1.382 μm, Sz 23.837 μm, 400.00 x 1026.778 x 23.936 μm
(B) Honeycrisp sample 1 site 2, Sa 1.667 μm, Sz 12.716 μm, 400.00 x 1016.005 x 13.187 μm
(C) Honeycrisp sample 1 site 3, Sa 1.613 μm, Sz 15.041 μm, 400.00 x 1035.894 x 17.566 μm
(D) Honeycrisp sample 1 site 4, Sa 2.412 μm, Sz 14.075 μm, 400.00 x 1006.060 x 14.074 μm
(E) Honeycrisp sample 1 site 5, Sa 1.384 μm, Sz 10.650 μm, 400.00 x 1009.375 x 10.649 μm.

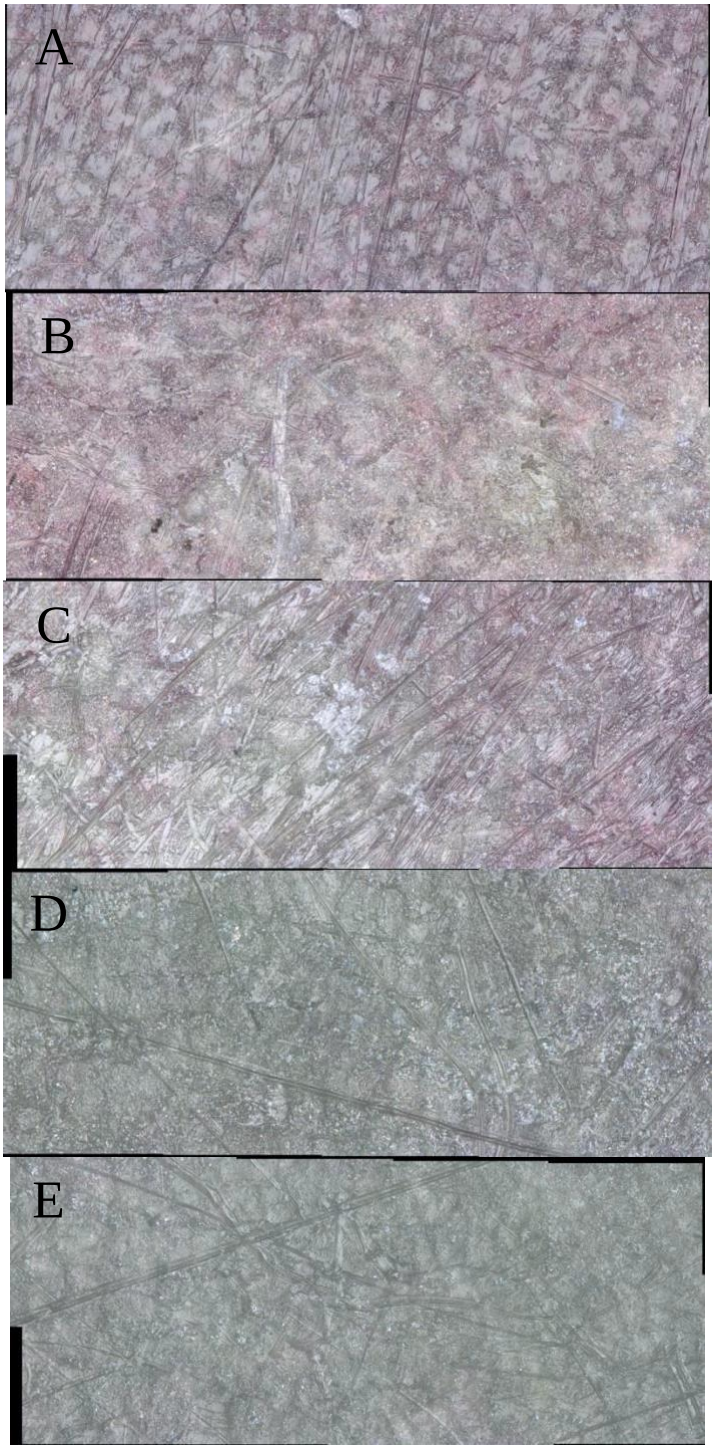

Figure 16. Honeycrisp sample 2 surface image taken with Keyence Digital Microscope VHX-6000.
(A) Honeycrisp sample 2 site 1, Sa 1.442 μm, Sz 14.337 μm, 400.00 x 1002.745 x 14.401 μm
(B) Honeycrisp sample 2 site 2, Sa 1.565 μm, Sz 28.377 μm, 400.00 x 1011.861 x 28.377 μm
(C) Honeycrisp sample 2 site 3, Sa 2.860 μm, Sz 16.022 μm, 400.00 x 1017.662 x 16.969 μm
(D) Honeycrisp sample 2 site 4, Sa 1.444 μm, Sz 13.220 μm, 400.00 x 1022.634 x 13.219 μm
(E) Honeycrisp sample 2 site 5, Sa 1.436 μm, Sz 9.918 μm, 400.00 x 1040.866 x 10.380 μm.

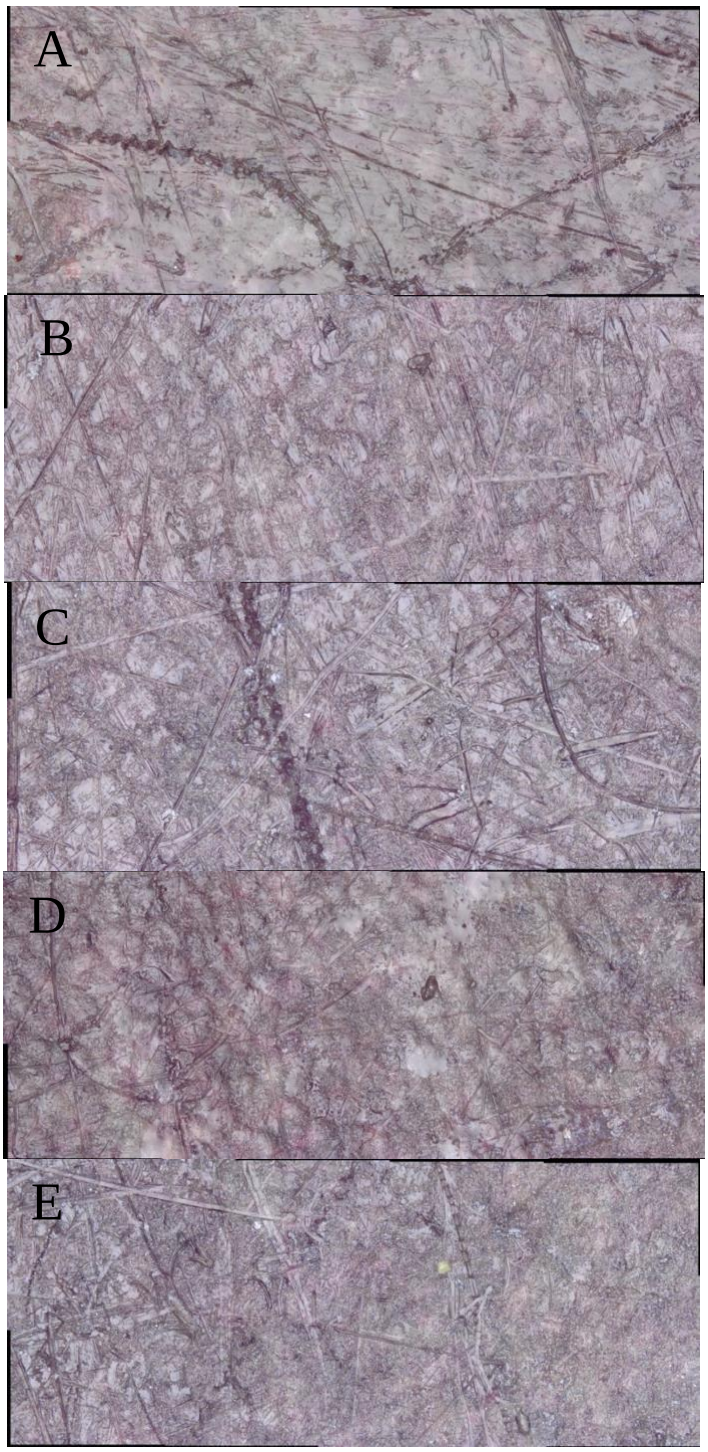

Figure 17. Honeycrisp sample 3 surface image taken with Keyence Digital Microscope VHX-6000.
(A) Honeycrisp sample 3 site 1, Sa 1.057 μm, Sz 8.107 μm, 400.00 x 1006.060 x 18.068 μm
(B) Honeycrisp sample 3 site 2, Sa 1.229 μm, Sz 8.032 μm, 400.00 x 1001.916 x 8.182 μm
(C) Honeycrisp sample 3 site 3, Sa 1.512 μm, Sz 11.717 μm, 400.00 x 1003.574 x 12.888 μm
(D) Honeycrisp sample 3 site 4, Sa 1.720 μm, Sz 20.076 μm, 400.00 x 1025.120 x 20.484 μm
(E) Honeycrisp sample 3 site 5, Sa 1.325 μm, Sz 11.926 μm, 400.00 x 1007.717 x 12.849 μm.

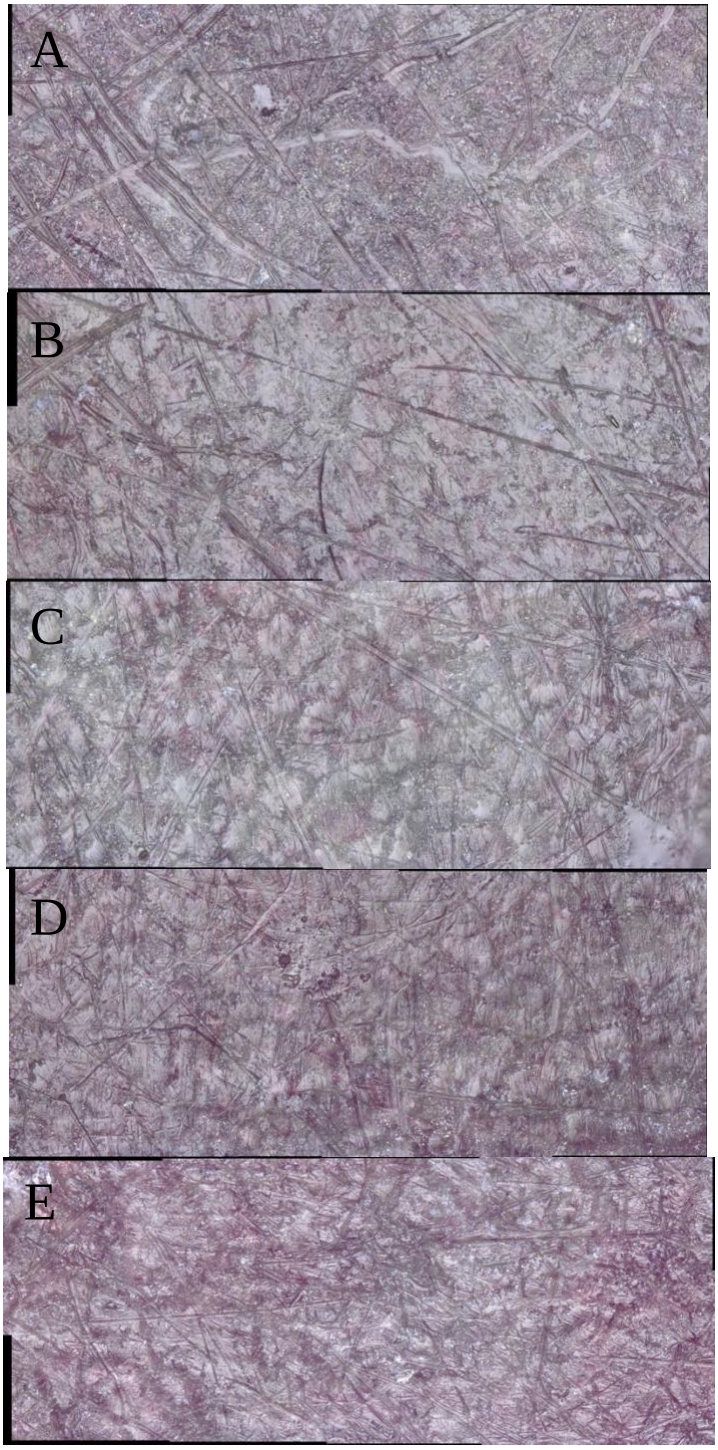

Figure 18. Honeycrisp sample 4 surface image taken with Keyence Digital Microscope VHX-6000.
(A) Honeycrisp sample 4 site 1, Sa 1.592 μm, Sz 11.963 μm, 400.00 x 1005.231 x 11.995 μm
(B) Honeycrisp sample 4 site 2, Sa 0.866 μm, Sz 13.103 μm, 400.00 x 1011.861 x 13.103 μm
(C) Honeycrisp sample 4 site 3, Sa 1.670 μm, Sz 20.010 μm, 400.00 x 1013.518 x 21.275 μm
(D) Honeycrisp sample 4 site 4, Sa 3.253 μm, Sz 18.821 μm, 400.00 x 1025.949 x 21.724 μm
(E) Honeycrisp sample 4 site 5, Sa 1.757 μm, Sz 14.235 μm, 400.00 x 1015.176 x 14.565 μm.

CHAPTER 5: THE IMPACT OF SHELLAC AND CARNAUBA APPLE FRUIT SURFACE COATINGS ON *LISTERIA MONOCYTOGENES* SURVIVAL

Sloniker, N., Alibashi, X., and Bergholz, T.

5.1 Abstract

To enhance quality, apple fruit are treated with coatings before they are shipped to market. The coating provides a barrier against water loss, reduces respiration, and improves the appearance of the fruit. Here we assess *L. monocytogenes* survival to determine the impact shellac and carnauba surface coatings have during storage in solution or applied to a contaminated surface. We observed a significant ($p < 0.05$) difference in *L. monocytogenes* log CFU/mL reductions for the surface coating solutions shellac and carnauba and their carrier solvent control solutions isopropyl alcohol and morpholine at 15 °C for up to 4 days of storage. After just 12 hours of storage, in the shellac surface coating solution, *L. monocytogenes* was no longer detectable, compared to the carnauba surface coating solution which still supported survival (2.4-4.0 log CFU/mL) after 14 days. *L. monocytogenes* populations were no longer detectable after 14 days of storage at 20 °C on shellac-coated filter membranes. *L. monocytogenes* populations were no longer detectable after 14 days of storage at 20 °C on shellac shellac-coated membranes. In comparison, all non-coated membranes had *L. monocytogenes* populations ranging from 5.72 to 7.77 log CFU/mL and some carnauba-coated samples had *L. monocytogenes* populations ranging from 2.4 to 4.0 log CFU/mL. The results from these experiments further support that shellac components could directly impact *L. monocytogenes* survival. As consumers and manufacturers look to natural food safety solutions, shellac and the compounds that make up shellac should be considered for further research.

5.2 Introduction

Listeria monocytogenes (*L. monocytogenes*) is a Gram-positive bacterial foodborne pathogen that causes an estimated 1,600 illnesses and 260 deaths annually in the United States alone. The populations most susceptible to listeriosis include newborns and infants, the elderly, pregnant women, and immunocompromised individuals (Centers for Disease Control and Prevention, 2021). Listeriosis can present in two forms a non-invasive form and an invasive form. The non-invasive, less severe form of listeriosis presents as a gastrointestinal illness, symptoms include fever, muscle aches, nausea, vomiting, and diarrhea. This form of listeriosis is primarily contracted by healthy individuals, who rarely contract the invasive form of listeriosis (Centers for Disease Control and Prevention, 2021). The symptoms of the invasive form of listeriosis include septicemia and meningitis caused by *L. monocytogenes* spread through the bloodstream. At-risk populations for the severe form of listeriosis include HIV patients, children under the age of 5, individuals over the age of 65, and pregnant individuals (Centers for Disease Control and Prevention, 2021).

L. monocytogenes is a heterogeneous species with 13 known serotypes, four phylogenetic lineages, as well as a growing number of clonal complexes (Cantinelli et al., 2013; Gorski et al., 2022). Further characterization of *L. monocytogenes* groups isolates into sequence types and clonal complexes. Multi-locus sequence typing (MLST) is a system that classifies *L. monocytogenes* isolates into sequence types and clonal complexes utilizing sequences of seven housekeeping genes. A clonal complex is a group of isolates that share six out of seven allelic sequences with at least one other sequence type in the group (V. Ferreira et al., 2014). The vast majority of listeriosis outbreaks are caused by just three *L. monocytogenes* serotypes, 1/2a, 1/2b, and 4b (Buchanan et al., 2016). Serotypes 1/2b and 4b are primarily

associated with lineage I and serotype 1/2a is primarily associated with lineage II (Orsi et al., 2011). Of the four phylogenetic lineages, I and II are associated with foodborne outbreaks while III and IV are seldom causes of human listeriosis (V. Ferreira et al., 2014).

Historically, food vehicles associated with *L. monocytogenes* have included processed, ready-to-eat foods including soft cheeses and sliced deli meats (Scallan et al., 2011). Conversely, in the past decade reports of *L. monocytogenes* contamination and prevalence on fresh produce have become more common. Fresh and ready-to-eat produce products are minimally processed, often consumed raw, and are primarily grown in an outdoor environment where *L. monocytogenes* can be present (Datta & Burall, 2018). While pre-harvest conditions can cause produce contamination, post-harvest contamination is the primary cause of *L. monocytogenes* outbreaks in fresh produce (Orsi et al., 2011).

Contamination of *L. monocytogenes* on apples was first identified as a concern in October 2014 when consumption of *L. monocytogenes*-contaminated caramel apples was traced to 35 cases of listeriosis across 12 states, including 34 hospitalizations, and one death (Buchanan, 2016; Centers for Disease Control and Prevention, 2021). Environmental testing along the supply chain confirmed two *L. monocytogenes* strains (CC01 and CC382), in the apple-packing facility matched the two clinical isolates (Buchanan et al., 2016). Concerning environmental contamination, the waxing unit operation, where edible coatings are applied, has been designated as a hotspot for *L. monocytogenes* during environmental sampling studies (Estrada et al., 2020; Ruiz-Llacsahuanga et al., 2021; Simonetti et al., 2021). These research findings parallel conversations conducted with apple industry members who highlighted the apple wax unit operation as a concern area for *Listeria* spp. (J. Kober, personal communication, February 26, 2021). The waxing unit operation is

inherently difficult to clean, and if the wax is not removed, it can build up and trap organisms leading to ineffective sanitation.

Edible coatings are a thin, edible layer of material applied to a product surface. Lipid-based coatings are most common for apples and are mainly used for their hydrophobic properties (Dhall, 2013; Pashova, 2023). The coating provides a barrier against water loss and improves the appearance of the fruit. The two most common lipid coatings, shellac, and carnauba are wax and resin-based. Shellac resin is secreted by the female lac bug (*Laccifer lacca*) and is primarily used for its glossy sheen in coatings for food, cosmetics, pharmaceuticals, and furniture (Dhall, 2013). Carnauba wax is a plant wax present on the surface of Brazilian palm (*Coernicia cerifera*) leaves and has a high melting point and is often used to create a humidity barrier. Applications of carnauba wax include coatings for food, cosmetics, automobiles, and furniture (Pashova, 2023).

Edible coatings have been found to enhance the quality and extend the shelf-life of apples (Raghav 2016); however, limited work has been done to determine the impact of commonly used commercial wax coatings on foodborne pathogens associated with fresh produce. The effect of fruit surface coatings on *Listeria monocytogenes* survival on apples was tested and after two months of storage and apples that had been previously waxed had a significantly higher *Listeria monocytogenes* survival compared to apples that had not been waxed (Macarisin et al., 2019). The study did not compare surface coating types and only used a shellac-based apple coating solution, while other formulas are used in industry (de Freitas et al., 2019). Here we assess *L. monocytogenes* survival to determine the impact shellac and carnauba surface coatings have during storage in solution or applied to a contaminated surface.

5.3 Materials and Methods

Bacterial strains and growth conditions. Fourteen strains of *L. monocytogenes* were used (Table 5). Seven of the strains were collected from the environment of apple packing facilities and were obtained from the Food Safety Laboratory at Cornell University (Cornell University; Wiedmann & Ivanek, 2021). The strains were taken from three different packing facilities and consisted of three different SigB allelic types: 64, 61, and 57. Seven additional *L. monocytogenes* strains were obtained from the laboratory of Dr. Sophia Kathariou at North Carolina State University. The strains were used based on relevance to apple-related hazards and other outbreaks as well as being used in previous work inoculating apples with *L. monocytogenes* (Sloniker et al., 2023).

Table 5. Panel of fourteen *Listeria monocytogenes* strains

Strain	Lineage	ST/CC	Source
FSL S11-0890	II	37/37	Top chain by waxer
FSL S11-1023	I	379/379	Puddle by floor wall juncture
FSL S11-1132	I	06/06	Drain in wax drying room
FSL S11-1290	I	554/554	Drain in wax drying room
FSL S11-1514	I	489/489	Drain under a brush bed
FSL S11-1613	II	37/37	Floor crack under waxer
FSL S11-1935	I	554/554	Cut section and top of leg on wax bed
4b1	I	145/02	Clinical isolate, 1962
H7858	I	06/06	Hot dog, 1998-99
2010L-1723	I	378/378	Celery, 2010
CFSAN023957	I	554/554	Mung bean sprouts, 2014
2014L-6680	I	01/01	Caramel apple, 2014-15
2014L-6695	I	382/382	Caramel apple, 2014-15
CFSAN073872	I	581/581	Apples, 2017

Fruit surface coating solutions and storage. Two edible pome coatings were chosen based on their primary ingredients and relevance to the apple industry. Both shellac- and carnauba- based coatings were obtained from a local apple packing house. The shellac-based coating used was Shield-Brite AP-40 (Pace International, Wapato, WA, USA) this coating uses isopropyl alcohol (16% [*v*/*v*]) as a solvent. The carnauba-based coating used was Prima Fresh 360 HS (Pace International, Wapato, WA, USA) this coating uses morpholine (1% [*v*/*v*]) as a solvent. The solvent concentration was informed by safety data sheets from Pace International. The coatings were then stored at room temperature and applied at full strength according to the manufacturer's instructions. Additionally, to ensure the primary solvent in the coatings did not impact bacteria survival, two controls were used to represent the primary solvent concentration in the coatings. Both a carnauba-based coating solvent control (1% [*v*/*v*] morpholine) and a shellac-based coating solvent control (16% [*v*/*v*] isopropyl alcohol) solutions were made with phosphate buffered saline (PBS) and stored at room temperature. To prepare for inoculation, 20 mL of shellac, carnauba, shellac solvent control, or carnauba solvent control were added to 50 mL Corning polypropylene centrifuge tubes (ThermoFisher Scientific, Waltham, MA, USA). About 12-24 h before inoculation tubes were stored at either 15 or 27 °C. These storage temperatures were selected based on the coldest and warmest temperatures fruit surface coating solutions experience in apple packing facilities (J. Kober, personal communication, March 10, 2023).

Inoculum preparation. *L. monocytogenes* stock cultures were stored at -80 °C in trypticase soy broth containing 0.6% (*w*/*v*) yeast extract (TSBYE, Neogen, Lansing, MI, USA) and 10% (*v*/*v*) glycerol (Sigma-Aldrich, Inc., St. Louis, MO, USA). Working cultures were prepared by streaking the frozen stock culture onto modified tryptic soy agar (mTSAYE)

containing 0.6% yeast extract (Neogen), 0.1% (*w*/*v*) esculin, and 0.5% (*w*/*v*) ferric ammonium citrate (Sigma-Aldrich) followed by incubation (37 °C, 48 h). A single colony was then transferred to 100 mL TSBYE and incubated (37 °C, 24 h). The inoculum suspensions were then centrifuged (8000 RPM, 10 min, 4 °C) and resuspended in 10 mL PBS. The final inoculum concentration was

~10^{10} CFU/mL.

Surface coating solution inoculation, storage, and sampling. The fourteen strain inoculation suspensions were then directly added via pipette to fourteen different tubes containing 20 mL each of a surface coating or solvent control solutions. After adding 200 uL of each inoculation suspension, all fourteen tubes were then vortexed and sampled. After the initial sampling (day 0), tubes were then returned to storage at 15 or 27 °C.

Inoculated coatings and controls were sampled immediately after inoculation (day 0), and further sampling of the stored samples took place after 12, 24, and 48 h, as well as 4, 7, and 14 d. Each sample was serially diluted in PBS and plated onto mTSAYE and *L. monocytogenes* colonies were enumerated after incubation (37 °C, 48 h).

Membrane inoculation, storage, and sampling. To replicate the spraying and drying process of fruit surface coatings, hydrophobic, 0.2 micron, 25 mm Polyester (PETE), membrane filters (Sterlitech, Washington, USA) were used to represent an apple surface. The membranes were inoculated with a cocktail of two strains from the caramel apple outbreak 2014L-6680 and 2014L-6695, these strains were chosen as they exhibited the highest survival in the surface coating solution inoculation trials. The membranes were also inoculated with a cocktail of two strains from the apple packinghouse environment FSL S11-1514 and FSL S11-1613, these strains were chosen as they exhibited the lowest survival in the surface coating

solution inoculation trials. The strains were similarly prepared as previously described, however, before inoculation, the strains 2014L-6680 and 2014L-6695 were mixed as a cocktail and FSL S11-1514 and FSL S11-1613 were mixed as a cocktail.

Each membrane was then placed in a sterile 6-Well Plate (ThermoFisher Scientific) inoculated with 100 uL of a cocktail and dried at room temperature in a biosafety cabinet for ~2 h. After complete drying, membranes were randomly assigned to either control, shellac, or carnauba treatments. The membranes to be coated were transported to a fume hood and sprayed with a fine mist sprayer from two inches away twice resulting in ~300 μL applied. The membranes were then dried at room temperature in a biosafety cabinet for ~30 m. Once dried, initial sampling (day 0) was conducted, and samples were then stored in Mylar Bags at either 4 or 20 °C. These storage temperatures were chosen as they represent refrigeration or room temperature storage of apples. Inoculated membranes were sampled after the coatings were dry (day 0), and further sampling of the stored samples took place after 7 and 14 d. Using sterile forceps, each membrane was then added to a 50 mL Corning polypropylene centrifuge tube with 20 mL of PBS and glass beads (2 mm, ThermoFisher Scientific). The tubes were then vortexed (60 s) and the sample was serially diluted in PBS and plated on mTSAYE and *L. monocytogenes* colonies were enumerated after incubation (37 °C, 48 h).

Apple fruit inoculation, storage, and sampling. Gala apples were chosen to be inoculated as they were previously indicated in apple-related *L. monocytogenes* outbreaks and recalls as well as previous work on *L. monocytogenes* survival on apples (Macarisin et al., 2019; Salazar et al., 2016; Sloniker et al., 2023). Apple fruit was collected from Sparta, MI, and stored at 4 °C before use. The apples were inoculated with a cocktail of two strains from the caramel apple outbreak 2014L-6680 and 2014L-6695, these strains were chosen as they exhibited the

highest survival in the surface coating solution inoculation trials. The strains were prepared as previously described, however before inoculation, the strains 2014L-6680 and 2014L-6695 were mixed as a cocktail.

Three 25 mm circles were drawn on each apple and each circle was inoculated with 50 uL of the cocktail before drying at room temperature in a biosafety cabinet for ~2 h. After complete drying, the apples were then randomly assigned to either control, shellac, or carnauba treatments. The apples to be coated were then transported to a fume hood and sprayed with a fine mist sprayer from two inches away twice resulting in ~300 μL applied. The apples were then dried at room temperature in a biosafety cabinet for ~30 m. Once dried, initial sampling (day 0) was conducted, samples were then stored at 4 °C. Inoculated circles were sampled after the coatings were dry (day 0), and further sampling of the stored apples took place after 7 and 14 d. Using a sterile cork borer (25 mm diameter) each circle was then sampled from the apple and the flesh was removed. Each apple skin circle was then added to a 50 mL Corning polypropylene centrifuge tube with 20 mL of PBS and glass beads (2 mm, ThermoFisher Scientific). The tubes were then vortexed (60 s) and the sample was appropriately diluted in PBS and plated on mTSAYE and *L. monocytogenes* colonies were enumerated after incubation (37 °C, 48 h).

Statistical Analysis. Three independent trials were conducted where fourteen *L. monocytogenes* strains were inoculated into four surface coating solutions and samples were stored at two storage temperatures. One sample was taken from each of the inoculated surface coating solutions for every sampling time. Analysis of covariance (ANCOVA) and post-hoc pairwise comparisons of means were used to determine statistical significance of the *L. monocytogenes* log CFU/mL between the *L. monocytogenes* strains, surface coating solutions,

and storage temperatures. Three independent trials were conducted where two *L. monocytogenes* cocktails inoculated membranes that were subsequently coated with two surface coatings and stored at two storage temperatures. Control samples were inoculated and not coated. One membrane was sampled for each treatment at every sampling time. Analysis of covariance (ANCOVA) and post- hoc pairwise comparisons were used to determine statistical significance between the *L. monocytogenes* cocktails, surface coating solutions, and storage temperatures. Three independent trials were conducted where one *L. monocytogenes* cocktail inoculated a 25 mm circle on an apple that was subsequently coated with one of two surface coatings and stored. Control samples were inoculated and not coated. One circle was sampled for each treatment at every sampling time. Students t-tests were used to determine statistical significance between the coating solutions on the apples. All statistical significance comparisons were conducted in RStudio Version 2023.06.1+524 (RStudio, Inc., Boston, MA, USA)

5.4 Results

Initial *L. monocytogenes* populations in surface coating and solvent control solutions. For the fourteen *L. monocytogenes* strains, the inoculum used to inoculate the surface coatings and solvent controls was on average 9.85±0.48 log CFU/mL. Immediately after inoculating the surface coatings and solvent controls, there was no significant ($p > 0.05$) difference in *L. monocytogenes* log CFU/mL reductions between the group of outbreak strains and the group of environmental strains, regardless of treatment. Moreover, there was a significant ($p < 0.01$) difference in *L. monocytogenes* log CFU/mL reductions when comparing storage temperatures (15 and 27 °C) of treatments shellac (3.86±0.93; 5.55±1.33), carnauba (3.94±0.70, 3.72±0.62), and isopropyl alcohol

(Figure 19 & 20).

Immediately after inoculation, surface coating solutions shellac and carnauba were significantly ($p < 0.001$) different when compared to their solvent control solutions isopropyl alcohol and morpholine for *L. monocytogenes* log CFU/mL reductions, regardless of storage temperature (Figure 19 & 20). For treatments stored at 15 °C there was no significant ($p > 0.05$) difference in *L. monocytogenes* log CFU/mL reductions between the surface coating solutions shellac (3.86±0.93) and carnauba (3.72±0.62). For treatments stored at 27 °C there was a significant ($p < 0.001$) difference in *L. monocytogenes* log CFU/mL populations between the surface coating solutions shellac (5.55±1.33) and carnauba (3.94±0.70)

Storage *L. monocytogenes* populations in surface coatings and solvent controls. After 12 hours of storage, surface coating solutions shellac and carnauba were significantly ($p < 0.001$) different from their solvent control solutions isopropyl alcohol and morpholine for *L. monocytogenes* log CFU/mL reductions regardless of temperature (Figure 19 & 20). *L.*

monocytogenes populations were also significantly ($p < 0.001$) different when comparing the surface coating solutions shellac and carnauba, regardless of temperature. Additionally, after just 12 hours of storage, *L. monocytogenes* populations were below the limit of detection (1.7 log CFU/mL) in all shellac surface coating solutions. There was also a significant ($p < 0.001$) difference in *L. monocytogenes* log CFU/mL reductions between storage temperatures (15 and 27 °C) for isopropyl alcohol (2.24±0.52; 7.13±1.02), carnauba (4.77±0.77; 6.10±1.16), and morpholine (2.89±0.61; 3.79±0.57).

After 1 day of storage at 15 °C, surface coating solutions shellac (8.11±0.40) and carnauba (5.05±0.84) were significantly ($p < 0.001$) different from their solvent control solutions isopropyl alcohol (2.71±0.77) and morpholine (3.21±0.58) for *L. monocytogenes* log CFU/mL reductions (Figure 19 & 20). After storage at 27 °C, surface coating solution carnauba (7.10±1.13) was significantly ($p < 0.001$) different from the solvent control solution morpholine (5.95±1.49). *L. monocytogenes* populations were also significantly ($p < 0.001$) different when comparing the surface coating solutions shellac and carnauba, regardless of temperature. Like the 12-hour samples, after 1 day there was also a significant ($p < 0.001$) difference in *L. monocytogenes* log CFU/mL reductions between storage temperatures (15 and 27 °C) for isopropyl alcohol (2.71±0.77; 7.96±0.66), carnauba (5.05±0.84; 7.10±1.13), and morpholine (3.21±0.58; 5.95±1.49).

After 2 days of storage at 15 °C, surface coating solutions shellac (8.11±0.40) and carnauba (5.49±0.94), were significantly ($p < 0.001$) different from their solvent control solutions isopropyl alcohol (3.69±1.43) and morpholine (3.61±0.65) (Figure 19 & 20). After

storage at 27 °C, surface coating solution carnauba (7.77±0.73) was significantly ($p < 0.001$) different from the solvent control solution morpholine (7.25±1.02). *L. monocytogenes* populations were also significantly (p

< 0.001) different when comparing the surface coating solutions shellac and carnauba, regardless of temperature. *L. monocytogenes* populations were below the limit of detection (1.7 log CFU/mL) in all isopropyl alcohol samples stored at 27 °C. Like the 12-hour and 1-day samples, after 2 days there was also a significant ($p < 0.001$) difference in *L. monocytogenes* log CFU/mL reductions between storage temperatures (15 and 27 °C) for isopropyl alcohol (3.69±1.43; 8.11±0.40),

carnauba (5.49±0.94; 7.77±0.73) and morpholine (3.61±0.65, 7.25±1.02).

After 4 days of storage at 15 °C surface coating solutions shellac (8.11±0.40) and carnauba (6.96±1.42), were significantly ($p < 0.001$) different from their solvent control solutions isopropyl alcohol (6.80±1.87) and morpholine (5.67±1.95) (Figure 19 & 20). After storage at 27 °C there was no significant ($p > 0.05$) difference in *L. monocytogenes* log CFU/mL reductions between the surface coating solutions and their solvent control solutions. There was also no significant ($p > 0.05$) difference in *L. monocytogenes* log CFU/mL reductions between the surface coating solutions shellac and carnauba. Like the previous storage samples, after 4 days there was also a significant ($p < 0.001$) difference in *L. monocytogenes* log CFU/mL reductions between storage temperatures (15 and 27 °C) for isopropyl alcohol (6.80±1.87; 8.11±0.40), carnauba (6.96±1.42;

7.95±0.51), and morpholine (5.67±1.95; 8.21±0.72).

After 7 days of storage there was no significant ($p > 0.05$) difference in *L. monocytogenes* log CFU/mL reductions between the surface coating solutions and their solvent

control solutions, regardless of storage temperature (Figure 19 & 20). There was also no significant ($p > 0.05$) difference in *L. monocytogenes* log CFU/mL reductions between the surface coating solutions shellac and carnauba, regardless of storage temperature. Additionally, there was a significant ($p > 0.05$) difference in *L. monocytogenes* log CFU/mL reductions between storage temperatures (15 and 27 °C) for carnauba (7.58±1.13; 8.11±0.40) and morpholine (7.26±1.51; 8.38±0.57)

After 14 days of storage at 15 °C there was no significant ($p > 0.05$) difference in *L. monocytogenes* log CFU/mL reductions between the surface coating solutions and their solvent control solutions, regardless of storage temperature (Figure 19 & 20). After storage at 27 °C there was a significant ($p < 0.05$) difference in *L. monocytogenes* log CFU/mL reductions between the surface coating solution carnauba (8.11±0.40) and morpholine (8.38±0.40). There was also no significant ($p > 0.05$) difference in *L. monocytogenes* log CFU/mL reductions between the surface coating solutions shellac and carnauba, regardless of storage temperature. *L. monocytogenes* populations were below the limit of detection (1.7 log CFU/mL) in all carnauba and morpholine samples stored at 27 °C. Following 14 days of storage there was no significant ($p < 0.05$) difference in *L. monocytogenes* log CFU/mL reductions between storage temperatures (15 and 27 °C), regardless of treatment.

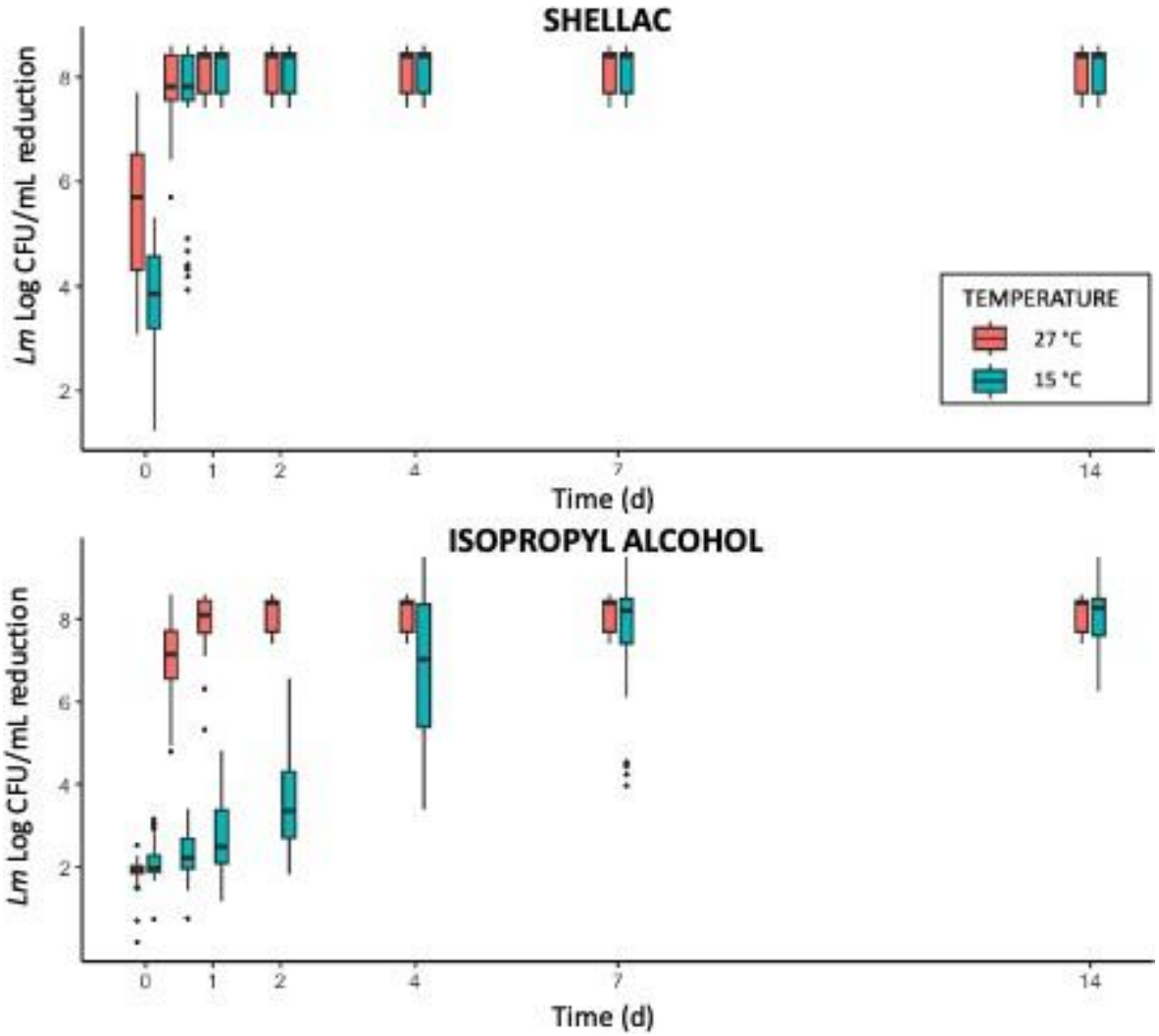

Figure 19. Reductions of *L. monocytogenes* populations in inoculated apple surface coating solutions stored at and 27 (red) and 15 (blue) °C. Reductions were determined as log CFU/mL and shown as boxplots. Surface coating solution shellac and solvent control solution 15% [*v*/*v*] isopropyl alcohol mixture were inoculated with fourteen *L. monocytogenes* strains.

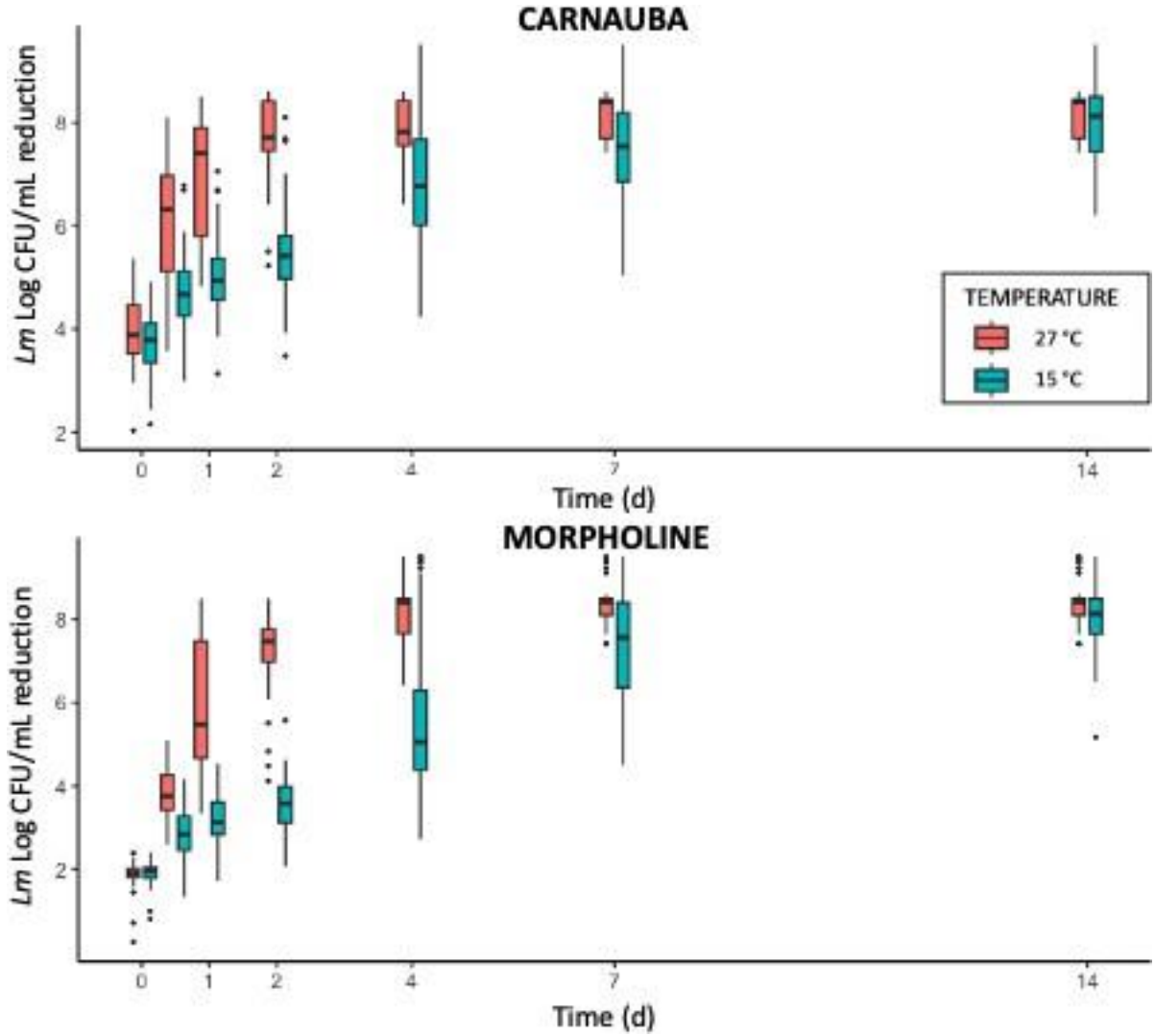

Figure 20. Reductions of *L. monocytogenes* populations in inoculated apple surface coating solutions stored at and 27 (red) and 15 (blue) °C. Reductions were determined as log CFU/mL and shown as boxplots. Surface coating solution carnauba and solvent control solution 1% [*v*/*v*] morpholine were inoculated with fourteen *L. monocytogenes* strains.

Initial *L. monocytogenes* populations on membrane filters. The two cocktails used to inoculate the membranes were on average 10.2±0.07 log CFU/mL. Immediately after inoculating and coating the membranes, there was a significant ($p < 0.001$) difference in *L. monocytogenes* log CFU/mL reductions when comparing shellac to control and carnauba to control (Figure 21). There was no signific ant ($p > 0.05$) difference in *L. monocytogenes* log CFU/mL reductions between the outbreak and environmental strains, regardless of treatment.

Storage *L. monocytogenes* populations on membrane filters. After 7 days of storage at 4 and 20 °C there was a significant ($p < 0.05$) difference in *L. monocytogenes* log CFU/mL reductions when comparing shellac (4.97±1.67; 6.24±1.59) to control (1.73±1.10; 2.00±0.31) and carnauba (3.71±0.77; 5.22±0.83) to control (1.73±1.10; 2.00±0.31) (Figure 21). Additionally, there was a significant ($p < 0.05$) difference in *L. monocytogenes* log CFU/mL reductions between storage temperatures (4 and 20 °C) for the carnauba coated (3.71±0.77; 5.22±0.83) samples. There was no significant ($p > 0.05$) difference in *L. monocytogenes* log CFU/mL reductions between the outbreak and environmental strains, regardless of treatment and temperature.

After 14 days of storage at 4 °C there was a significant ($p < 0.05$) difference in *L. monocytogenes* log CFU/mL reductions between all treatments shellac (6.46±1.30), carnauba (3.40±0.27), and control (1.99±0.54) (Figure 21). Furthermore, after 14 days of storage in 20 °C there was a significant ($p < 0.001$) difference in *L. monocytogenes* log CFU/mL reductions when comparing shellac (7.30±0.00) to control (2.48±1.87) and carnauba (7.05±0.41) to control (2.48±1.87). Like day 7, there was a significant ($p < 0.05$) difference in *L. monocytogenes* log CFU/mL reductions between storage temperatures (4 and 20 °C) for the carnauba (3.40±0.27; 7.05±0.38) coated samples. There was no significant ($p > 0.05$) difference in *L.*

monocytogenes log CFU/mL reductions between the outbreak and environmental strains, regardless of treatment and temperature.

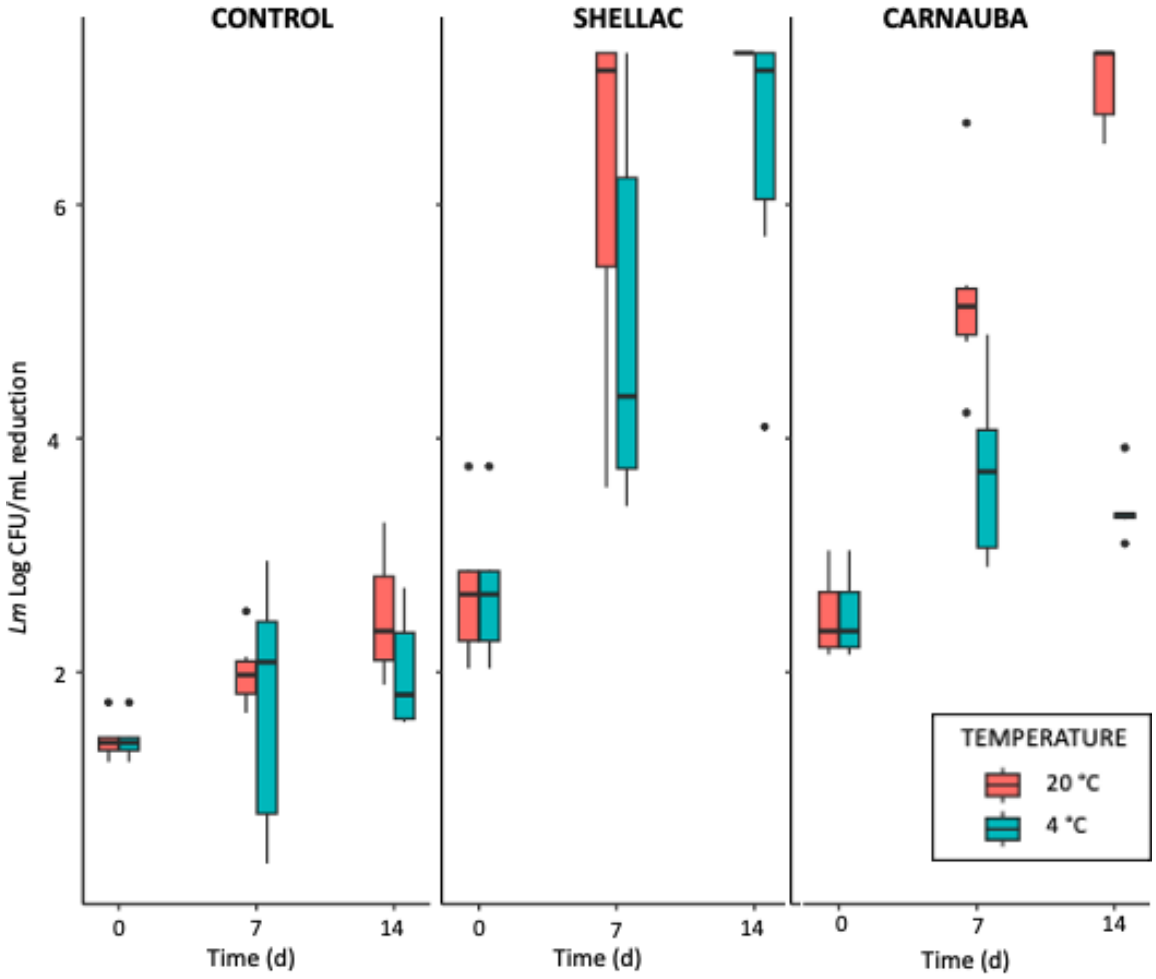

Figure 21. Reductions of *L. monocytogenes* populations on inoculated filter membranes stored at 4 °C (blue) and 20 °C (red). Reductions were determined as log CFU/mL and shown as boxplots. Filter membranes were inoculated with two strain cocktails of outbreak and environmental strains, then treated with shellac and carnauba surface coating solutions.

Initial L. monocytogenes populations on apples. Each 25 mm circle was inoculated with 9.70±0.13 log CFU/circle. Immediately after inoculating and coating the apples, there was a significant ($p < 0.01$) difference in *L. monocytogenes* log CFU/mL reductions when comparing shellac (6.89±0.17) to control (6.02±0.14) (Figure 22). There was also a significant ($p < 0.05$) difference in *L. monocytogenes* log CFU/mL reductions when comparing shellac (6.89±0.17) to carnauba (6.24±0.33).

Storage *L. monocytogenes* populations on apples. After 14 days of storage at 4 °C there was a significant ($p < 0.01$) difference in *L. monocytogenes* log CFU/mL reductions when comparing control (6.29±0.64) to shellac (8.90±0.17) and carnauba (8.80±0.35) (Figure 22).

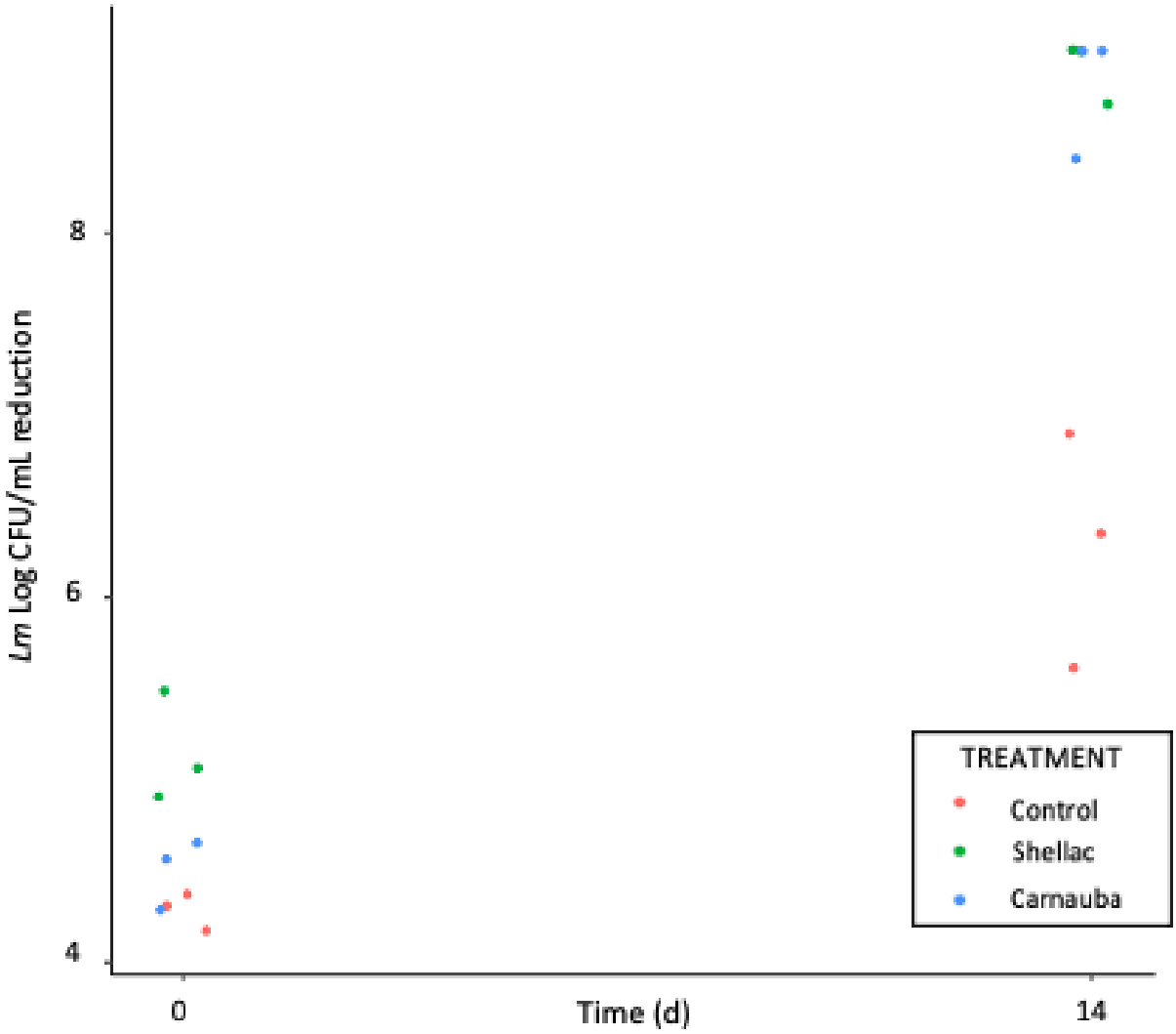

Figure 22. Reductions of *L. monocytogenes* populations on inoculated Gala apples stored at 4 °C. Reductions were determined as log CFU/mL and shown as scatterplots. Apples were inoculated with a two-strain cocktail of outbreak strains, then treated with shellac and carnauba surface coating solutions.

5.5 Discussion

In this study, storage temperatures impacted the reduction of *L. monocytogenes.* For example, at 15 °C there was no significant ($p > 0.05$) difference in *L. monocytogenes* log CFU/mL reductions between shellac and carnauba however, at 27 °C there was a significant ($p < 0.001$) difference. In a study on *L. monocytogenes* survival in four storage citrus waxes, survival was significantly higher at storage temperature 4 °C compared to 22 °C (Sheng et al., 2023). Over two weeks, apples stored at 1, 4, or 10 °C had lower *L. monocytogenes* reductions (0.2-0.3 log CFU/apple) compared to apples stored at 22 °C (0.5-1.2 log CFU/apple) (Sheng et al., 2017). These studies support that the survival of *L. monocytogenes* is impacted by storage temperatures. We also observed a significantly ($p < 0.05$) higher reduction of *L. monocytogenes* log CFU/mL for the surface coating solutions shellac and carnauba compared to their carrier solvent control solutions isopropyl alcohol and morpholine at 15 °C for up to 4 days of storage. These differences lead us to believe that the decrease *in L. monocytogenes* was not attributed to the solvents and shows potential for shellac coating components to directly impact *L. monocytogenes* survival.

After just 12 hours of storage in the shellac surface coating solution, *L. monocytogenes* was no longer detectable, compared to the carnauba surface coating solution which still supported survival (2.4-4.0 log CFU/mL) after 14 days. *L. monocytogenes* populations were no longer detectable after 14 days of storage at 20 °C on shellac-coated membranes. In comparison, all non- coated membranes had *L. monocytogenes* populations (5.72-7.77 log CFU/mL), and some carnauba-coated samples had *L. monocytogenes* populations (2.4-4.0 log CFU/mL). The results from these experiments further support that shellac components could directly impact *L. monocytogenes* survival. Shellac is a natural resin composed of aleuritic

acid and cyclic terpene acids that can vary but often include aleuritic acid, shellolic acid, jalaric acid, laccijalaric acid, laksholic acid, laccishellolic acid, or laccilaksholic acid (Yuan et al., 2021). One study has tested the impact of different shellac formulations on the survival of *Escherichia coli*, *Enterobacter aerogenes*, and *Klebsiella pneumonia* in liquid solution (McGuire & Hagenmaier, 2001). A shellac coating solution with 5% morpholine reduced *Escherichia coli* and *Klebsiella pneumonia* from 6 log CFU/mL to below the limit of detection (5 CFU/mL) in three hours at 24 °C (McGuire & Hagenmaier, 2001). In a study on novel natural composite films, a disk diffusion method was used to determine the impact of varying amounts of shellac on the survival of *Bacillus mycoides*, *Escherichia coli*, and *Candida albicans* (Mohamed et al., 2019). They found the inhibition zones increased in size as the amount of shellac increased in the composite films. For the 8% shellac films, there was a 12 mm inhibition zone for *Escherichia coli*, and in the 14% shellac films, *Escherichia coli*, *Bacillus mycoides*, and *Candida albicans*, had inhibition zones 17, 10, and 10 mm, respectively (Mohamed et al., 2019). While there are already many uses for shellac, there is little research on its food safety applications.

Moreover, while many studies have been done on the use of shellac and carnauba coatings to improve the quality of many fresh produce products, few have been done on the ability of coatings to improve the safety of fresh produce products (Bai et al., 2002; Hagenmaier, 2005; Zhou et al., 2015). In a study on the effect of a shellac surface coating on *L. monocytogenes* survival on apples, after two months of storage, apples that had been coated with shellac had significantly ($p < 0.05$) more survival compared to apples that had not been treated with wax (Macarisin et al., 2019). However, for Red Delicious and Fuji apples, the *L. monocytogenes* populations in uncoated apples were significantly ($p < 0.05$) higher compared

to the coated apples for 30 days. These data support our observations for *L. monocytogenes* reductions on coated samples. Typically, when apples are removed from storage, they are processed, and shipped to the supermarket the same day. Based on this timeline, the short-term impact of surface coatings on *L. monocytogenes* reduction is most critical to food safety. Very few studies have been done on food-based antimicrobial uses for shellac coatings, most research on shellac and carnauba coatings focus on the non-food antimicrobial uses (Yuan et al., 2021). This is surprising as shellac and carnauba have been Generally Recognized as Safe (GRAS) for decades and are already being used to coat many food products (Yuan et al., 2021). The Food and Drug Administration (FDA) defines an antimicrobial agent as "used to control microorganisms such as bacteria, viruses, fungi, protozoa, or other microorganisms in or on food or food contact articles (Center for Food Safety and Applied Nutrition, 2021)." To submit an antimicrobial agent request to the FDA, they recommend efficacy data show a quantifiable reduction of the target pathogen in the samples treated with the antimicrobial agent when compared to the negative controls (Center for Food Safety and Applied Nutrition, 2021). As consumers and manufacturers look to natural food safety solutions, shellac and the compounds that make up shellac should be considered for further research.

5.6 Acknowledgements

We would like to acknowledge the contribution of Jamie Kober for the valuable information and supplies. As well as Corrine Kamphuis, Avery Evans, and Shaney Rump for their helpful contribution to lab work.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

The purpose of this work was to contribute knowledge about *Listeria monocytogenes* survival on fresh, whole apples as impacted by fruit attributes and post-harvest conditions. As fresh produce contamination and outbreaks have increased due to better detection methods, it is important to replicate the storage and processing environments as closely as possible to determine where and why the current environment is supporting the survival of *L. monocytogenes*. It is also important to look to the current circumstances for practical intervention strategies.

Even though apples were stored at low temperatures in air and controlled atmosphere storage for up to seven months, *L. monocytogenes* was able to survive at infectious levels. Survival was impacted by bacterial growing conditions as *L. monocytogenes* that had grown on a surface survived at higher populations compared to the liquid-grown cells. This information is important as *L. monocytogenes* outbreaks attributed to fresh produce are almost always caused by environmental contamination. These data show that the ability of *L. monocytogenes* to grow on a surface may improve its ability to attach to fresh produce in the processing and packing environment. This information should be used to inform future research for *L. monocytogenes* survival on fresh produce as currently, most survival studies use liquid-grown cells which do not represent the growth conditions *L. monocytogenes* are experiencing in the food processing environment. This work not only addressed the growing conditions of *L. monocytogenes* strains but also used strains that related to fresh produce outbreaks.

Even with multiple post-harvest conditions tested during the long-term storage study, differences in survival between apple cultivars were seen. Specifically, Gala apples had lower survival of *L. monocytogenes* compared to Granny Smith and Honeycrisp apples. In chapter

three we further investigated both Gala and Honeycrisp cuticular waxes for possible differences in chemical compounds. Alkanes were the only compound group where Gala apples had more of each compound in comparison to Honeycrisp apples. Nonacosane was higher in Gala apples and has been shown to have antimicrobial activity against Gram-positive bacteria (El-Hawary, 2018).

At present there is already a market for using the naturally occurring defensive compounds in fresh produce coatings applied during processing. Apeel Sciences is a company that was inspired by the cuticular wax layer on plants as they developed a coating to extend the shelf life of fresh produce. In the cuticular waxes, while there may not have been one compound responsible for reducing *L. monocytogenes* populations, there were many compounds identified to have antimicrobial properties. Triterpenoids were the most abundant compound in the waxes and should be further researched for their reported antimicrobial properties against human pathogens. There is potential for antimicrobial compounds present in the naturally occurring coating of fresh produce to be applied in greater concentrations with commercially applied coatings.

For apple fruit, there are two main commercially applied coatings, shellac and carnauba, these coatings had never previously been assessed for their impact on foodborne pathogens associated with fresh produce outbreaks. After just 12 hours of storage in the shellac surface coating solution, *L. monocytogenes* was no longer detectable, compared to the carnauba surface coating solution which still supported survival after 14 days. We also observed a significant ($p < 0.05$) difference in *L. monocytogenes* log CFU/mL reductions for the surface coating solutions shellac and carnauba and their carrier solvent control solutions isopropyl alcohol and morpholine. These differences lead us to believe that the decrease *in L. monocytogenes* was not

attributed to the solvents and shows potential for coating components to directly impact *L. monocytogenes* survival. Specifically, these results further support that shellac components could directly impact *L. monocytogenes* survival.

The ability of the shellac coating solution to reduce *L. monocytogenes* counts by 8 log CFU/mL in less than 12 hours is intriguing and future research should study the antimicrobial activity of the individual chemical components of shellac, specifically the compounds in the highest concentration, aleuritic acid, shellolic acid, and jalaric acid. Additionally, contact times less than 12 hours should be assessed as immediate contact resulted in 4-6 log CFU/mL reductions of *L. monocytogenes*. When shellac was applied to a contaminated surface, reduction was still observed over a longer period.

The research presented here shows how fresh produce post-harvest conditions support the survival *of L. monocytogenes* and it highlights potential practical intervention strategies in the current conditions. Future work should focus on utilizing fresh produce surface coatings as an opportunity for food safety interventions.

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APPENDIX 1: HARVEST YEAR 2019 *LISTERIA MONOCYTOGENES* LOG CFU/APPLE SURVIVAL

Table 6. Michigan apples harvest year 2019 *Listeria monocytogenes* log CFU/apple survival

Cell growth	Cultivar	Store	Time (d) 0	Time (d) 7	Time (d) 14	Time (d) 30	Time (d) 60	Time (d) 90	Time (d) 180	Time (d) 210
Biofilm	Gala	Air	6.08	5.97	6.73	5.84	6.35	5.63		
Biofilm	Gala	Air	6.37	6.34	6.21	6.02	5.61	5.93		
Biofilm	GS	Air	6.17	5.99	5.66	6.05	5.67	5.58		
Biofilm	GS	Air	5.77	6.12	5.75	6.34	5.68	5.29		
Biofilm	HC	Air	5.8	6.74	6.56	6.42	5.7	5.82		
Biofilm	HC	Air	6.48	6.74	6.32	6.48	5.78	5.96		
Planktonic	HC	Air	5.76	3.90	3.70	3.35	3.37		2.4	
Planktonic	HC	Air	5.58	3.98	4.39	2.46	2.32		1.62	
Planktonic	HC	CA	5.76	4.00	4.39	3.52	3.71		3.32	2.77
Planktonic	HC	CA	5.58	4.54	5.07	2.32	3.57		4.28	3.46
Biofilm	Gala	CA	6.08	6.64	6.42	6.1	6.33	6.03		5.52
Biofilm	Gala	CA	6.37	6.33	6.32	6.29	6.41	5.62		5.57
Biofilm	GS	CA	6.17	6.25	6.39	5.51	5.66	5.38		5.58
Biofilm	GS	CA	5.77	6.2	6.24	5.51	5.94	5.87		5.69
Biofilm	HC	CA	5.8	6.88	6.93	6.43	6.4	6.32		6.02
Biofilm	HC	CA	6.48	6.65	6.8	6.5	6.47	6.06		6.2
Planktonic	Gala	Air	4.92	3.40	5.60	4.30	4.48	4.08		2.82
Planktonic	Gala	Air	4.88	4.34	5.51	4.81	3.95	4.45		2.87
Planktonic	GS	Air	3.22	6.65	4.03	2.77	4.71	2.52		2.1
Planktonic	GS	Air	4.40	6.84	4.15	3.97	3.37	3.45		2.96
Planktonic	Gala	CA	4.92	2.92	6.05	3.71	4.17	2.62		
Planktonic	Gala	CA	4.88	3.52	5.72	4.03	4.01	2.92		
Planktonic	GS	CA	3.22	6.71	4.92	3.92	2.98	2.90		
Planktonic	GS	CA	4.40	6.40	2.92	3.97	3.07	3.86		

Table 7. Pennsylvania apples harvest year 2019 *Listeria monocytogenes* log CFU/apple survival

Cell growth	Cultivar	Store	Time (d) 0	Time (d) 7	Time (d) 14	Time (d) 30	Time (d) 60	Time (d) 90	Time (d) 180	Time (d) 210
Planktonic	Gala	Air	5.86	5.82	5.61	4.11	3.4	3.53		
Planktonic	Gala	Air	5.26	6.15	6.34	3.37	2.62	3.23		
Planktonic	GS	Air	3.22	6.6	3.92	3.02	2.62	3.23		
Planktonic	GS	Air	3.22	6.81	3.92	2.82	2.92	1.92		
Planktonic	HC	Air	5.89	3.73	2.62	2.98	3.62		3.86	
Planktonic	HC	Air	5.29	4.47	2.92	4.12	2.92		3.79	
Planktonic	Gala	CA	5.86	3.92	6.68	2.82	3.62	3.47		1.32
Planktonic	Gala	CA	5.26	3.22	6.35	3.34	3.62	3.35		1.32
Planktonic	GS	CA	3.22	6.37	3.92	3.41	2.73	3.65		1.92
Planktonic	GS	CA	3.22	6.09	3.62	3.33	3.16	2.4		2.57
Planktonic	HC	CA	5.89	4.02	3.1	5.2	3.63		3.6	2.22
Planktonic	HC	CA	5.29	4.29	3.98	4.84	3.86		3.16	2.66

Table 8. Washington apples harvest year 2019 *Listeria monocytogenes* log CFU/apple survival

Cell growth	Cultivar	Store	Time (d) 0	Time (d) 7	Time (d) 14	Time (d) 30	Time (d) 60	Time (d) 180	Time (d) 210
Planktonic	Gala	Air	4.58	2.62	3.1	3.32	1.92	1.92	
Planktonic	Gala	Air	4.65	2.92	2.62	1.62	3.62	2.62	
Planktonic	GS	Air	4.14	2.92	2.62	2.46	5.68	1.62	
Planktonic	GS	Air	4.24	2.62	3.66	1.62	4.35	2.9	
Planktonic	HC	Air	3.96	2.62	2.62	2.32	2.62	1.32	
Planktonic	HC	Air	5	2.62	3.62	1.92	3.1	1.32	
Planktonic	Gala	CA	4.58	2.92	6.12	3.95	4.14	2.46	4.35
Planktonic	Gala	CA	4.65	2.62	3.62	3.24	3.95	3.14	3.47
Planktonic	GS	CA	4.14	2.92	3.1	4.25	5.37	3.42	4.7
Planktonic	GS	CA	4.24	2.92	2.62	4.22	4.07	2.66	3.44
Planktonic	HC	CA	3.96	3.82	2.62	2.92	1.92	3.98	3.11
Planktonic	HC	CA	5	3.52	3.62	2.57	1.92	2.62	2.32

APPENDIX 2: HARVEST YEAR 2020 *LISTERIA MONOCYTOGENES* LOG CFU/APPLE SURVIVAL

Table 9. Michigan apples harvest year 2020 *Listeria monocytogenes* log CFU/apple survival

Cell growth	Cultivar	Storage	Time (d) 0	Time (d) 7	Time (d) 14	Time (d) 30	Time (d) 60	Time (d) 90	Time (d) 120	Time (d) 150	Time (d) 180
Biofilm	Gala	Air	5.92	5.68	5.35	4.84	4.62	4.70			
Biofilm	Gala	Air	6.29	5.79	5.61	5.38	4.28	4.58			
Biofilm	GS	Air	5.95	4.91	5.22	5.31	5.48	4.47			
Biofilm	GS	Air	5.96	5.35	5.83	5.30	5.09	4.61			
Biofilm	HC	Air	6.66	5.58	5.95	5.32	5.53	5.77			
Biofilm	HC	Air	6.50	5.94	5.92	5.79	5.00	4.90			
Biofilm	Gala	CA	5.92	5.64	4.83	5.35	4.87	4.43	4.40	4.12	3.77
Biofilm	Gala	CA	6.29	5.62	4.45	5.23	4.82	4.35	4.68	4.15	4.10
Biofilm	GS	CA	5.95	5.02	5.45	5.31	5.08	4.82	4.91	4.23	4.59
Biofilm	GS	CA	5.96	5.28	5.54	5.56	5.66	4.55	4.72	3.98	4.28
Biofilm	HC	CA	6.66	6.07	5.85	5.73	4.54	5.32	4.59	4.33	4.34
Biofilm	HC	CA	6.50	6.05	5.72	5.59	4.56	5.38	5.17	4.86	3.31
Planktonic	Gala	Air	5.29	3.57	3.15	3.43	2.73	2.10			
Planktonic	Gala	Air	5.29	3.10	2.22	2.96	2.70	2.90			
Planktonic	GS	Air	5.48	5.07	3.99	4.15	4.92	3.73			
Planktonic	GS	Air	5.54	4.52	3.82	4.06	3.91	3.90			
Planktonic	HC	Air	5.24	4.52	4.39	3.76	4.35	3.86			
Planktonic	HC	Air	4.85	4.54	4.57	3.38	4.78	4.62			
Planktonic	Gala	CA	5.29	3.96	3.02	2.96	2.22	3.03	2.73	2.62	1.62
Planktonic	Gala	CA	5.29	3.32	3.63	3.61	3.19	2.46	1.92	2.32	2.10
Planktonic	GS	CA	5.48	4.56	3.84	4.01	3.60	4.27	4.63	3.37	4.36
Planktonic	GS	CA	5.54	5.41	4.19	4.54	3.93	4.14	3.43	3.37	3.30
Planktonic	HC	CA	5.24	4.45	4.93	4.41	4.04	4.53	4.18	3.15	3.11
Planktonic	HC	CA	4.85	4.76	4.93	4.16	4.32	4.02	4.35	3.30	4.62

Table 10. Pennsylvania apples harvest year 2020 *Listeria monocytogenes* log CFU/apple survival

Cell growth	Cultivar	Storage	Time (d) 0	Time (d) 7	Time (d) 14	Time (d) 30	Time (d) 60	Time (d) 90	Time (d) 120	Time (d) 150	Time (d) 180
Planktonic	Gala	Air	5.32	2.10	2.85	2.57	2.57	2.40			
Planktonic	Gala	Air	4.32	2.10	1.62	2.10	1.62	1.62			
Planktonic	GS	Air	5.49	3.92	1.92	2.57	2.40	2.90			
Planktonic	GS	Air	5.45	3.92	3.29	2.22	2.40	1.62			
Planktonic	HC	Air	5.41	4.48	3.96	2.62	2.62	1.62			
Planktonic	HC	Air	5.40	4.66	4.07	3.22	3.10	2.32			
Planktonic	Gala	CA	5.32	2.70	2.40	2.70	1.92	1.62	1.62	1.62	1.62
Planktonic	Gala	CA	4.32	3.07	2.40	1.62	2.22	1.62	1.62	1.62	1.62
Planktonic	GS	CA	5.49	1.62	3.11	2.70	2.32	1.62	1.62	2.10	1.62
Planktonic	GS	CA	5.45	4.22	3.12	1.62	1.62	1.62	1.62	1.62	1.62
Planktonic	HC	CA	5.41	4.23	4.40	2.92	2.88	2.57	1.62	2.22	2.62
Planktonic	HC	CA	5.40	4.59	3.40		3.20	2.46	1.62	1.62	2.10

Table 11. Washington apples harvest year 2020 *Listeria monocytogenes* log CFU/apple survival

Cell growth	Cultivar	Storage	Time (d) 0	Time (d) 7	Time (d) 14	Time (d) 30	Time (d) 60	Time (d) 90	Time (d) 120	Time (d) 150	Time (d) 180
Planktonic	Gala	Air	4.33	2.46	1.62	2.98	2.66	4.15			
Planktonic	Gala	Air	4.08	3.08	1.62	1.62	3.26	3.39			
Planktonic	GS	Air	5.21	3.92	3.63	3.74	4.33	3.77			
Planktonic	GS	Air	5.44		3.76	4.35	4.52	4.73			
Planktonic	HC	Air	4.69	3.64		3.37	4.90	4.88			
Planktonic	HC	Air	5.12	4.02	3.51	3.34	5.01	4.77			
Planktonic	Gala	CA	4.33	2.90	2.22	2.22	3.86	2.10	1.62	2.46	2.40
Planktonic	Gala	CA	4.08	3.03	2.70		3.80	3.19	1.62	2.88	2.32
Planktonic	GS	CA	5.21	2.92	3.05	3.27	3.76	4.01	5.20	4.79	4.05
Planktonic	GS	CA	5.44	3.22	3.62	3.62	3.38	3.88	3.38	3.39	3.79
Planktonic	HC	CA	4.69	3.80	3.75	3.00		4.28	3.16	3.22	3.84
Planktonic	HC	CA	5.12	4.20	3.15			4.74	3.00	3.56	4.12

APPENDIX 3: APPLE WAX COMPOSITION UG/CM^2 OF PEEL

Table 12. Apple wax alcohol composition ug/cm^2 of peel

Sample	Hexacosanol	Nonacosan-10-ol	Octacosanol	**Tetracosanol**	Triacontanol
Gala waxes 1	4.949	0.321	0.465	3.573	0.346
Gala waxes 2	11.747	0.367	0.889	5.595	0.420
Gala waxes 3	8.084	0.335	0.830	4.470	0.401
Gala waxes 4	5.435	0.332	0.474	3.773	0.345
Gala waxes 5	5.656	0.315	0.475	3.598	0.337
Gala waxes 6	5.093	0.310	0.469	3.540	0.338
Gala waxes 7	6.072	0.326	0.608	3.840	0.357
Gala waxes 8	5.275	0.342	0.445	3.650	0.335
Honeycrisp waxes 11	17.777	11.245	1.400	6.370	0.504
Honeycrisp waxes 12	6.862	5.789	0.699	3.790	0.369
Honeycrisp waxes 13	5.125	4.513	0.617	3.488	0.365
Honeycrisp waxes 14	7.402	7.074	0.687	3.965	0.399
Honeycrisp waxes 16	6.771	5.010	0.613	3.898	0.379
Honeycrisp waxes 17	5.478	3.109	0.484	3.615	0.356
Honeycrisp waxes 18	5.397	5.945	0.521	3.583	0.383
Honeycrisp waxes 19	3.310	0.344	0.346	3.058	0.311

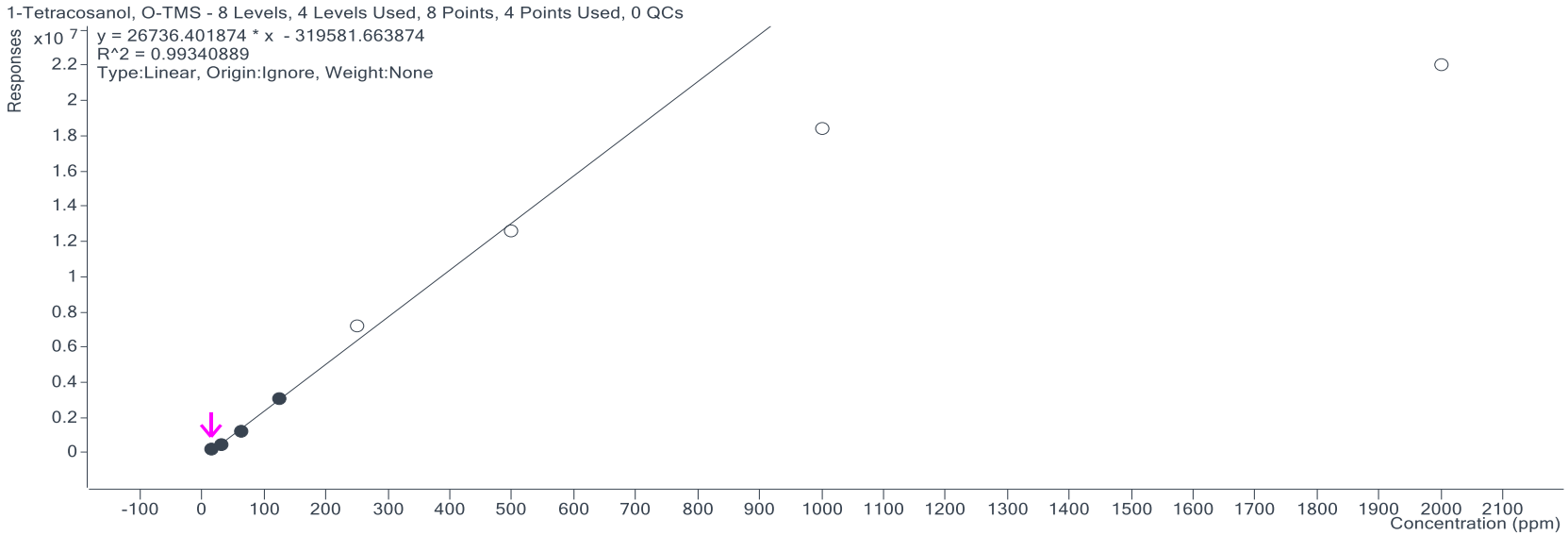

Figure 23. 1-Tetracosanol standard curve.

Table 13. Apple wax aldehyde composition ug/cm^2 of peel

Sample	Hexadecanal	Triacontanal
Gala waxes 1	< LOD	8.943
Gala waxes 2	< LOD	2.974
Gala waxes 3	< LOD	2.974
Gala waxes 4	< LOD	5.573
Gala waxes 5	< LOD	7.426
Gala waxes 6	< LOD	7.296
Gala waxes 7	< LOD	7.050
Gala waxes 8	< LOD	2.974
Honeycrisp waxes 11	< LOD	2.974
Honeycrisp waxes 12	< LOD	5.526
Honeycrisp waxes 13	< LOD	6.873
Honeycrisp waxes 14	< LOD	7.958
Honeycrisp waxes 16	< LOD	5.864
Honeycrisp waxes 17	< LOD	2.974
Honeycrisp waxes 18	< LOD	7.874
Honeycrisp waxes 19	< LOD	9.343

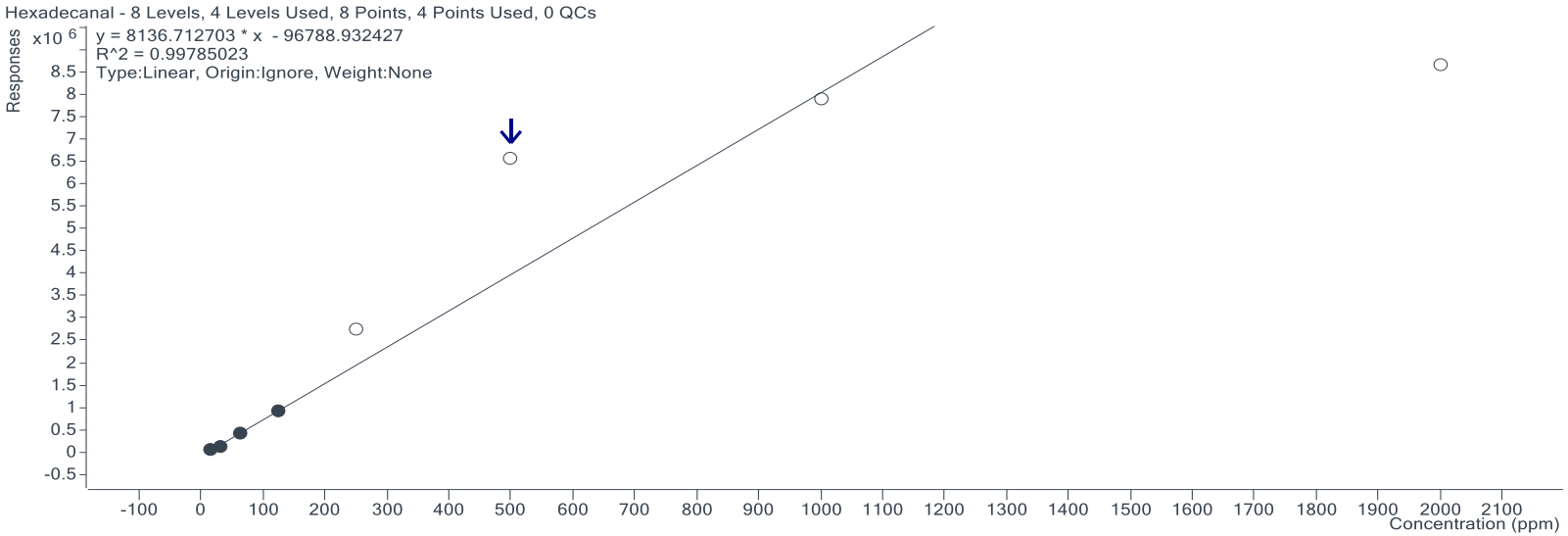

Figure 24. Hexadecanal standard curve.

Table 14. Apple wax alkane composition ug/cm^2 of peel

Sample	Heptacosane	Nonacosane
Gala waxes 1	36.313	1.797
Gala waxes 2	15.750	10.391
Gala waxes 3	20.263	2.220
Gala waxes 4	13.510	1.842
Gala waxes 5	20.280	1.828
Gala waxes 6	16.085	2.097
Gala waxes 7	19.373	2.306
Gala waxes 8	7.650	3.002
Honeycrisp waxes 11	17.698	58.560
Honeycrisp waxes 12	13.805	5.130
Honeycrisp waxes 13	12.000	5.530
Honeycrisp waxes 14	12.593	8.844
Honeycrisp waxes 16	10.703	7.583
Honeycrisp waxes 17	7.873	6.716
Honeycrisp waxes 18	12.538	6.266
Honeycrisp waxes 19	12.890	1.635

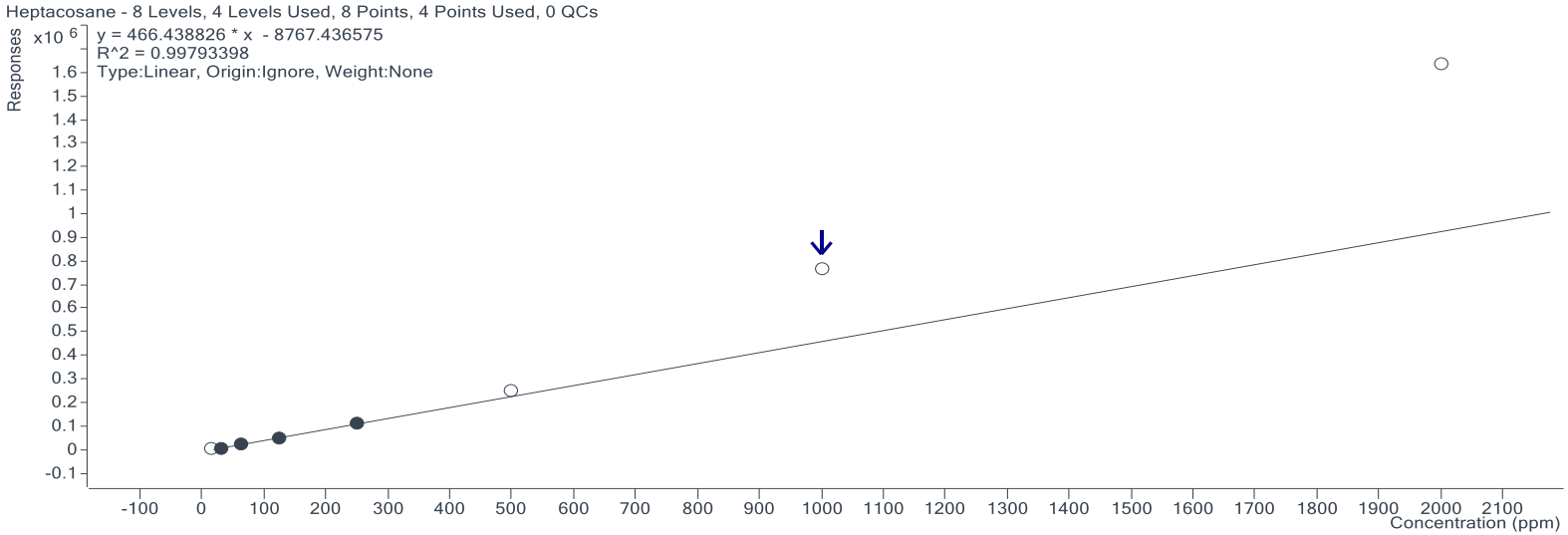

Figure 25. Heptacosane standard curve.

Table 15. Apple wax alkene composition ug/cm^2 of peel

Sample	Alpha Farnesene
Gala waxes 1	< LOD
Gala waxes 2	< LOD
Gala waxes 3	< LOD
Gala waxes 4	< LOD
Gala waxes 5	< LOD
Gala waxes 6	< LOD
Gala waxes 7	< LOD
Gala waxes 8	< LOD
Honeycrisp waxes 11	1.021
Honeycrisp waxes 12	0.932
Honeycrisp waxes 13	1.055
Honeycrisp waxes 14	1.246
Honeycrisp waxes 16	1.168
Honeycrisp waxes 17	0.785
Honeycrisp waxes 18	1.198
Honeycrisp waxes 19	0.746

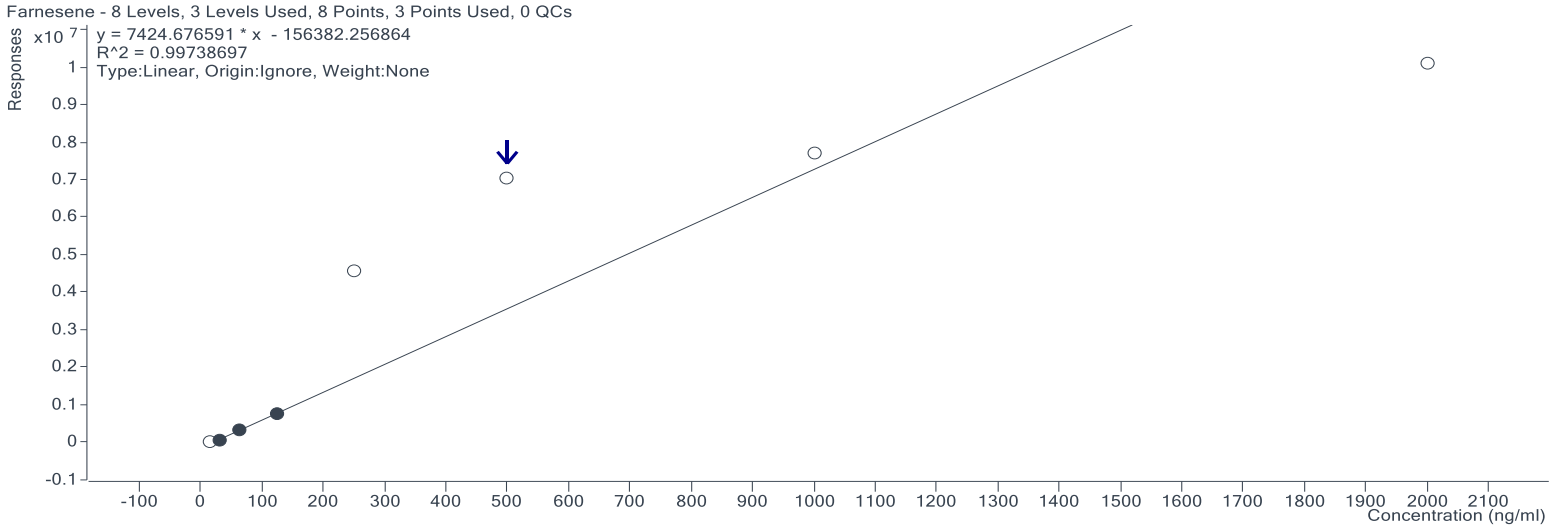

Figure 26. Farnesene standard curve.

Table 16. Apple wax fatty acid composition ug/cm^2 of peel

Sample	Hexacosanoic acid	Octacosanoic acid	Palmitic acid	Stearic acid	Tetradecanoic acid	Triacontanoic acid
Gala waxes 1	2.732	2.925	2.800	< LOD	< LOD	2.716
Gala waxes 2	2.816	2.991	3.561	< LOD	< LOD	2.855
Gala waxes 3	2.857	3.265	2.816	< LOD	< LOD	2.836
Gala waxes 4	2.757	2.916	2.705	< LOD	< LOD	2.749
Gala waxes 5	2.821	3.022	2.683	< LOD	< LOD	2.750
Gala waxes 6	2.760	2.976	2.708	< LOD	< LOD	2.785
Gala waxes 7	2.862	3.175	2.701	< LOD	< LOD	2.872
Gala waxes 8	2.678	2.762	2.765	< LOD	< LOD	2.721
Honeycrisp waxes 11	3.140	3.758	3.476	< LOD	< LOD	3.247
Honeycrisp waxes 12	2.807	3.140	2.730	< LOD	< LOD	2.911
Honeycrisp waxes 13	2.764	3.185	2.707	< LOD	< LOD	2.947
Honeycrisp waxes 14	2.805	3.319	2.707	< LOD	< LOD	3.024
Honeycrisp waxes 16	2.782	3.137	2.706	< LOD	< LOD	2.903
Honeycrisp waxes 17	2.723	2.984	2.729	< LOD	< LOD	2.841
Honeycrisp waxes 18	2.788	3.247	2.686	< LOD	< LOD	3.067
Honeycrisp waxes 19	2.746	2.831	2.659	< LOD	< LOD	2.632

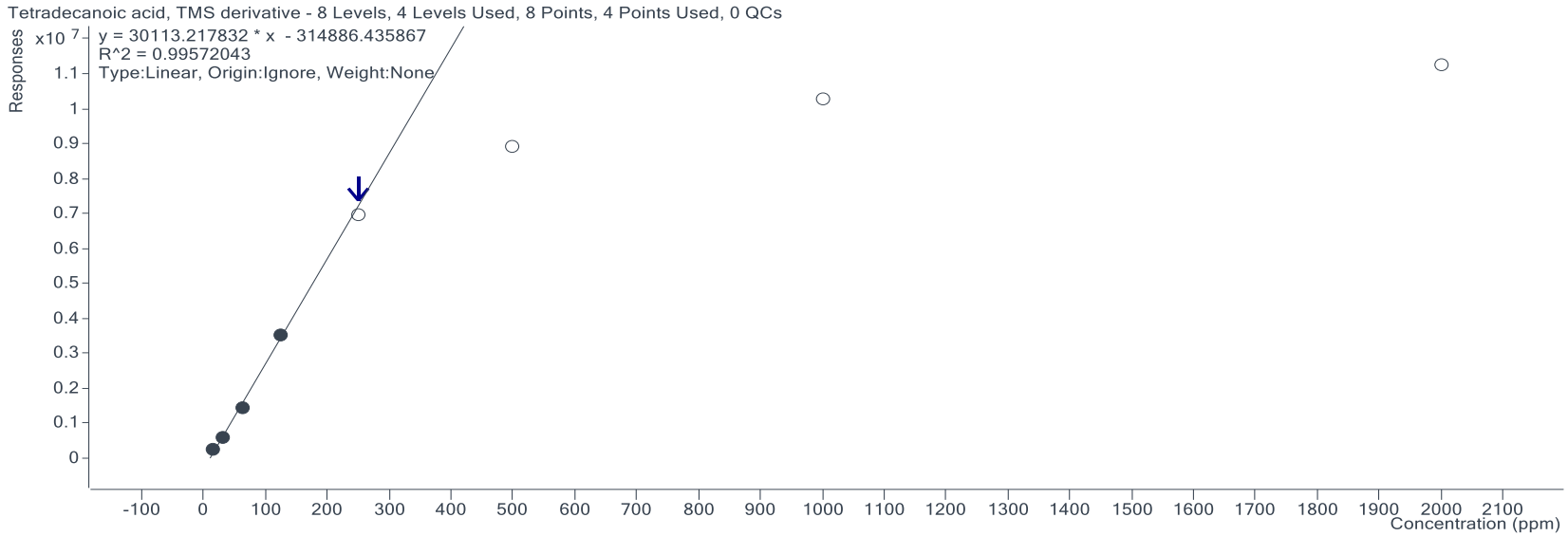

Figure 27. Tetradecanoic acid standard curve.

Table 17. Apple wax triterpenoid composition ug/cm^2 of peel

Sample	Oleanolic acid	Ursolic	Uvaol
Gala waxes 1	7.423	7.187	6.538
Gala waxes 2	25.243	41.564	7.036
Gala waxes 3	9.090	8.879	6.890
Gala waxes 4	7.928	7.369	6.904
Gala waxes 5	7.718	7.313	6.698
Gala waxes 6	8.610	8.387	6.675
Gala waxes 7	9.468	9.224	6.826
Gala waxes 8	10.913	12.010	6.770
Honeycrisp waxes 11	88.815	234.239	7.271
Honeycrisp waxes 12	16.263	20.521	6.961
Honeycrisp waxes 13	16.993	22.122	7.057
Honeycrisp waxes 14	25.045	35.376	7.248
Honeycrisp waxes 16	20.560	30.333	7.068
Honeycrisp waxes 17	18.125	26.864	6.804
Honeycrisp waxes 18	19.270	25.066	7.095
Honeycrisp waxes 19	< LOD	6.538	6.538

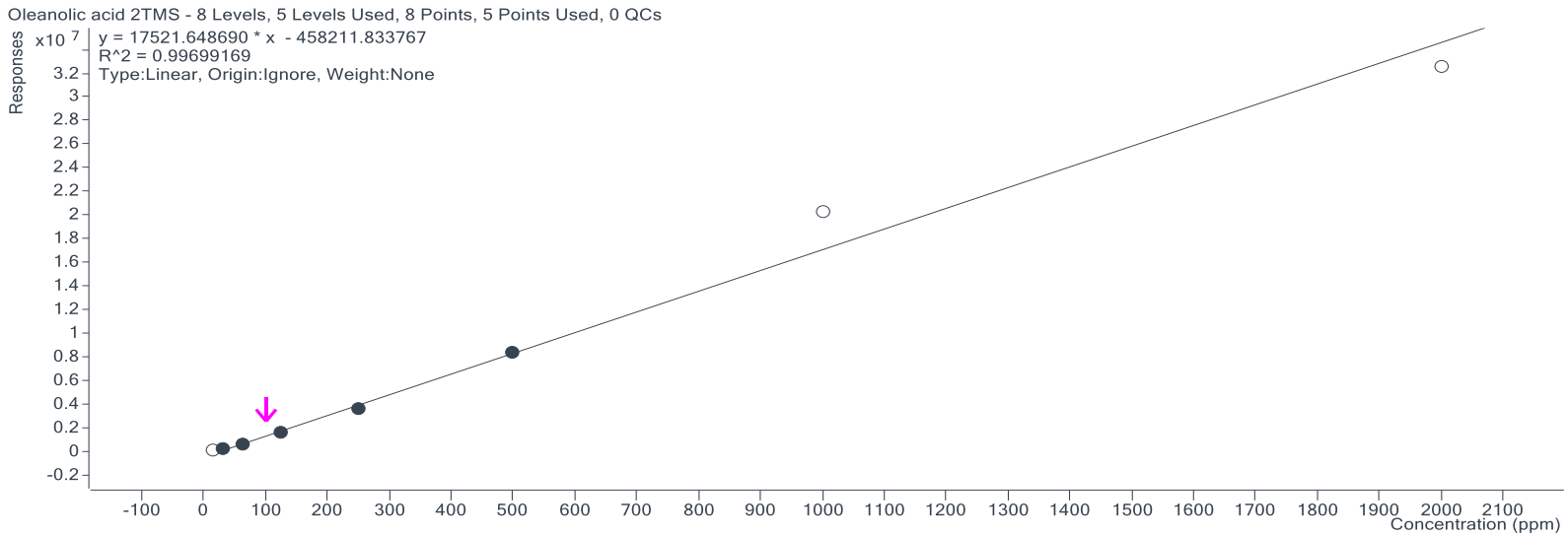

Figure 28. Oleanolic acid standard curve.

APPENDIX 4: APPLE FRUIT SURFACE COATING SOLUTIONS *LISTERIA* MONOCYTOGENES LOG CFU/ML DECREASE

Table 18. Trial 1 shellac apple fruit surface coating solution *Listeria monocytogenes* log cfu/mL decrease

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
CFSAN023957	15	4.18	4.18	8.4	8.4	8.4	8.4	8.4
CFSAN073872	15	4.31	4.31	8.42	8.42	8.42	8.42	8.42
2010L-1723	15	3.93	3.93	8.45	8.45	8.45	8.45	8.45
2014L-6680	15	4.91	4.91	8.47	8.47	8.47	8.47	8.47
2014L-6695	15	4.41	4.41	7.41	7.41	7.41	7.41	7.41
4b1	15	4.67	4.67	8.1	8.1	8.1	8.1	8.1
H7858	15	4.34	4.34	8.42	8.42	8.42	8.42	8.42
CFSAN023957	27	6.45	6.45	8.4	8.4	8.4	8.4	8.4
CFSAN073872	27	7.42	7.42	8.42	8.42	8.42	8.42	8.42
2010L-1723	27	5.7	5.7	8.45	8.45	8.45	8.45	8.45
2014L-6680	27	7.17	7.17	8.47	8.47	8.47	8.47	8.47
2014L-6695	27	6.41	6.41	7.41	7.41	7.41	7.41	7.41
4b1	27	7.1	7.1	8.1	8.1	8.1	8.1	8.1
H7858	27	7.42	7.42	8.42	8.42	8.42	8.42	8.42

Table 18 (cont'd).

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
FSL S11-0890	15	5.31	8.5	8.5	8.5	8.5	8.5	8.5
FSL S11-1023	15	4.9	8.5	8.5	8.5	8.5	8.5	8.5
FSL S11-1132	15	5.1	8.5	8.5	8.5	8.5	8.5	8.5
FSL S11-1290	15	5.16	8.6	8.6	8.6	8.6	8.6	8.6
FSL S11-1514	15	4.67	8.5	8.5	8.5	8.5	8.5	8.5
FSL S11-1613	15	5.06	8.4	8.4	8.4	8.4	8.4	8.4
FSL S11-1935	15	5.14	8.5	8.5	8.5	8.5	8.5	8.5
FSL S11-0890	27	7.3	8.5	8.5	8.5	8.5	8.5	8.5
FSL S11-1023	27	6.3	8.5	8.5	8.5	8.5	8.5	8.5
FSL S11-1132	27	6.37	8.5	8.5	8.5	8.5	8.5	8.5
FSL S11-1290	27	6.52	8.6	8.6	8.6	8.6	8.6	8.6
FSL S11-1514	27	7.04	8.5	8.5	8.5	8.5	8.5	8.5
FSL S11-1613	27	6.59	8.4	8.4	8.4	8.4	8.4	8.4
FSL S11-1935	27	6.05	8.5	8.5	8.5	8.5	8.5	8.5

Table 19. Trial 2 shellac apple fruit surface coating solution *Listeria monocytogenes* log cfu/mL decrease

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
CFSAN023957	15	2.87	8.4	8.4	8.4	8.4	8.4	8.4
CFSAN073872	15	4.38	8.42	8.42	8.42	8.42	8.42	8.42
2010L-1723	15	3.86	8.45	8.45	8.45	8.45	8.45	8.45
2014L-6680	15	3.52	8.47	8.47	8.47	8.47	8.47	8.47
2014L-6695	15	3.15	7.41	7.41	7.41	7.41	7.41	7.41
4b1	15	4.58	8.1	8.1	8.1	8.1	8.1	8.1
H7858	15	3.85	8.42	8.42	8.42	8.42	8.42	8.42
CFSAN023957	27	3.89	8.4	8.4	8.4	8.4	8.4	8.4
CFSAN073872	27	5.1	8.42	8.42	8.42	8.42	8.42	8.42
2010L-1723	27	4.13	8.45	8.45	8.45	8.45	8.45	8.45
2014L-6680	27	4.37	8.47	8.47	8.47	8.47	8.47	8.47
2014L-6695	27	7.41	7.41	7.41	7.41	7.41	7.41	7.41
4b1	27		8.1	8.1	8.1	8.1	8.1	8.1
H7858	27	3.62	8.42	8.42	8.42	8.42	8.42	8.42

Table 19 (cont'd).

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
FSL S11-0890	15	2.6	7.66	7.66	7.66	7.66	7.66	7.66
FSL S11-1023	15	2.57	7.67	7.67	7.67	7.67	7.67	7.67
FSL S11-1132	15	2.94	7.71	7.71	7.71	7.71	7.71	7.71
FSL S11-1290	15	2.98	8.15	8.15	8.15	8.15	8.15	8.15
FSL S11-1514	15	3.17	7.69	7.69	7.69	7.69	7.69	7.69
FSL S11-1613	15	3.6	7.64	7.64	7.64	7.64	7.64	7.64
FSL S11-1935	15	3.16	7.83	7.83	7.83	7.83	7.83	7.83
FSL S11-0890	27	4.79	7.66	7.66	7.66	7.66	7.66	7.66
FSL S11-1023	27	3.87	7.67	7.67	7.67	7.67	7.67	7.67
FSL S11-1132	27	4.11	7.71	7.71	7.71	7.71	7.71	7.71
FSL S11-1290	27	4.06	8.15	8.15	8.15	8.15	8.15	8.15
FSL S11-1514	27	7.09	7.69	7.69	7.69	7.69	7.69	7.69
FSL S11-1613	27	4.31	7.64	7.64	7.64	7.64	7.64	7.64
FSL S11-1935	27	4.09	7.83	7.83	7.83	7.83	7.83	7.83

Table 20. Trial 3 shellac apple fruit surface coating solution *Listeria monocytogenes* log cfu/mL decrease

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
CFSAN023957	15	3.59	8.4	8.4	8.4	8.4	8.4	8.4
CFSAN073872	15	1.23	8.42	8.42	8.42	8.42	8.42	8.42
2010L-1723	15	3.69	8.45	8.45	8.45	8.45	8.45	8.45
2014L-6680	15	3.57	8.47	8.47	8.47	8.47	8.47	8.47
2014L-6695	15	1.74	7.41	7.41	7.41	7.41	7.41	7.41
4b1	15	3.06	8.1	8.1	8.1	8.1	8.1	8.1
H7858	15	3.25	8.42	8.42	8.42	8.42	8.42	8.42
CFSAN023957	27	4.5	8.4	8.4	8.4	8.4	8.4	8.4
CFSAN073872	27	4.67	8.42	8.42	8.42	8.42	8.42	8.42
2010L-1723	27	4.54	8.45	8.45	8.45	8.45	8.45	8.45
2014L-6680	27	4.15	8.47	8.47	8.47	8.47	8.47	8.47
2014L-6695	27	3.08	7.41	7.41	7.41	7.41	7.41	7.41
4b1	27	5.52	8.1	8.1	8.1	8.1	8.1	8.1
H7858	27	3.67	8.42	8.42	8.42	8.42	8.42	8.42

Table 20 (cont'd).

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
FSL S11-0890	15	3.59	7.81	7.81	7.81	7.81	7.81	7.81
FSL S11-1023	15	3.78	7.78	7.78	7.78	7.78	7.78	7.78
FSL S11-1132	15	4.24	7.41	7.41	7.41	7.41	7.41	7.41
FSL S11-1290	15	3.92	7.67	7.67	7.67	7.67	7.67	7.67
FSL S11-1514	15	4.77	7.71	7.71	7.71	7.71	7.71	7.71
FSL S11-1613	15	4.55	7.53	7.53	7.53	7.53	7.53	7.53
FSL S11-1935	15	3.67	7.69	7.69	7.69	7.69	7.69	7.69
FSL S11-0890	27	5.83	7.81	7.81	7.81	7.81	7.81	7.81
FSL S11-1023	27	6.44	7.78	7.78	7.78	7.78	7.78	7.78
FSL S11-1132	27	6.33	7.41	7.41	7.41	7.41	7.41	7.41
FSL S11-1290	27	5.42	7.67	7.67	7.67	7.67	7.67	7.67
FSL S11-1514	27	7.71	7.71	7.71	7.71	7.71	7.71	7.71
FSL S11-1613	27	5.81	7.53	7.53	7.53	7.53	7.53	7.53
FSL S11-1935	27	5.36	7.69	7.69	7.69	7.69	7.69	7.69

Table 21. Trial 1 isopropyl alcohol apple fruit surface coating solution *Listeria monocytogenes* log cfu/mL decrease

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
CFSAN023957	15	1.84	2.97	4.82	6.5	6.42	8.4	8.4
CFSAN073872	15	1.87	2.85	3.68	6.52	5.64	6.25	6.25
2010L-1723	15	1.92	2.87	3.68	6.55	5.67	7.58	7.58
2014L-6680	15	1.93	2.73	3.7	6.57	7.64	8.47	8.47
2014L-6695	15	2.27	2.92	3.41	5.51	7.41	7.41	7.41
4b1	15	1.97	2.87	3.43	6.2	6.75	8.1	8.1
H7858	15	1.96	2.79	3.72	6.52	8.42	8.42	8.42
CFSAN023957	27	1.91	7.1	8.4	8.4	8.4	8.4	8.4
CFSAN073872	27	1.93	6.56	8.42	8.42	8.42	8.42	8.42
2010L-1723	27	2.01	6.81	8.45	8.45	8.45	8.45	8.45
2014L-6680	27	1.99	8.29	8.47	8.47	8.47	8.47	8.47
2014L-6695	27	2.27	7.41	7.41	7.41	7.41	7.41	7.41
4b1	27	1.89	7.27	8.1	8.1	8.1	8.1	8.1
H7858	27	2.02	8.42		8.42	8.42	8.42	8.42

Table 21 (cont'd).

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
FSL S11-0890	15	2.12	3.41	4.09	4.75	7.07	8.5	8.5
FSL S11-1023	15	2.06	2.22	2.49	3.35	5.2	8.2	8.5
FSL S11-1132	15	1.97	2.32	3.23	4.82	8.5	8.5	8.5
FSL S11-1290	15	2.11	2.46	3.4	4.66	8.6	8.6	8.6
FSL S11-1514	15	1.85	2.71	3.38	4.28	5.27	8.5	8.5
FSL S11-1613	15	1.97	2.93	3.77	4.31	5.83	8.4	8.4
FSL S11-1935	15	1.94	2.22	3.01	4.28	8.2	8.5	8.5
FSL S11-0890	27	2.14	8.5	8.5	8.5	8.5	8.5	8.5
FSL S11-1023	27	2.02	7.42	8.5	8.5	8.5	8.5	8.5
FSL S11-1132	27	2.06	6.77	8.5	8.5	8.5	8.5	8.5
FSL S11-1290	27	2.03	8.6	8.6	8.6	8.6	8.6	8.6
FSL S11-1514	27		8.5	8.5	8.5	8.5	8.5	8.5
FSL S11-1613	27	1.92	7.15	8.4	8.4	8.4	8.4	8.4
FSL S11-1935	27	1.93	8.5	8.5	8.5	8.5	8.5	8.5

Table 22. Trial 2 isopropyl alcohol apple fruit surface coating solution *Listeria monocytogenes* log cfu/mL decrease

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
CFSAN023957	15	1.99	2.29	2.65	3.5	8.1	7.4	7.1
CFSAN073872	15	2.1	2.13	2.53	2.72	3.52	4.24	7.12
2010L-1723	15	2.28	2.35	2.84	3.18	4.91	7.97	6.67
2014L-6680	15	2.11	2.49	3.05	3.81	8.17	8.17	7.47
2014L-6695	15	2.38	2.68	3.38	3.65	7.41	7.41	6.81
4b1	15	2.96	2.41	2.89	3.46	5.38	6.8	6.62
H7858	15	3.15	2.24	3	4.09	8.42	8.42	6.82
CFSAN023957	27	1.88	8.4	8.4	8.4	8.4	8.4	8.4
CFSAN073872	27	1.98	6.79	8.42	8.42	8.42	8.42	8.42
2010L-1723	27	2.1	8.45	8.45	8.45	8.45	8.45	8.45
2014L-6680	27	2.22	8.47	8.47	8.47	8.47	8.47	8.47
2014L-6695	27	2.52	7.41	7.41	7.41	7.41	7.41	7.41
4b1	27	2.2	7.1	8.1	8.1	8.1	8.1	8.1
H7858	27	2.05	8.42	8.42	8.42	8.42	8.42	8.42

Table 22 (cont'd).

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
FSL S11-0890	15	2.83	1.97	1.82	2.49	3.71	3.96	7.66
FSL S11-1023	15	2.91	2.19	2.18	2.62	4.15	7.67	7.67
FSL S11-1132	15	2.59	1.75	2.07	3.17	7.23	6.11	7.71
FSL S11-1290	15	3.02	2.26	2.39	2.91	8.15	8.15	8.15
FSL S11-1514	15	2.7	2.07	1.98	2.71	4.19		7.69
FSL S11-1613	15	3.06	2.21	2.08	2.7	3.39	4.54	7.64
FSL S11-1935	15	3.04	1.97	2.09	2.77	6.99	7.83	7.83
FSL S11-0890	27	1.84	6.62	7.66	7.66	7.66	7.66	7.66
FSL S11-1023	27	2	6.08	7.67	7.67	7.67	7.67	7.67
FSL S11-1132	27	1.47	6.81	7.71	7.71	7.71	7.71	7.71
FSL S11-1290	27	1.9	5.53	8.15	8.15	8.15	8.15	8.15
FSL S11-1514	27	1.6	7.69	7.69	7.69	7.69	7.69	7.69
FSL S11-1613	27	1.85	7.64	7.64	7.64	7.64	7.64	7.64
FSL S11-1935	27	1.9	7.53	7.83	7.83	7.83	7.83	7.83

Table 23. Trial 3 isopropyl alcohol apple fruit surface coating solution *Listeria monocytogenes* log cfu/mL decrease

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
CFSAN023957	15	1.76	1.82	2.19	3.36	6.62	8.4	8.1
CFSAN073872	15	1.78	1.93	2.26	2.22	4.12	4.43	8.42
2010L-1723	15	1.87		2.27	2.41	4.15	4.47	8.45
2014L-6680	15	1.93		2.42	3.42	6.26	8.47	8.47
2014L-6695	15	0.73	0.75	1.16	1.81	5.39	6.81	7.41
4b1	15	2.04	2.11	2.21	2.75	6.16	8.1	8.1
H7858	15	2.06	2.04	2.49	3.61	7.22	8.42	8.42
CFSAN023957	27	1.78	6.4	8.4	8.4	8.4	8.4	8.4
CFSAN073872	27	1.74	6.48	6.31	8.42	8.42	8.42	8.42
2010L-1723	27	1.88	5.88	7.11	8.45	8.45	8.45	8.45
2014L-6680	27	1.91		8.47	8.47	8.47	8.47	8.47
2014L-6695	27	0.69	4.79	5.32	7.41	7.41	7.41	7.41
4b1	27	2.13	5.8	8.1	8.1	8.1	8.1	8.1
H7858	27	0.17	4.94	8.42	8.42	8.42	8.42	8.42

Table 23 (cont'd).

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
FSL S11-0890	15	1.91	1.81	1.95	2.05	9.51	9.51	9.51
FSL S11-1023	15	1.95	1.77	2.09	2.3	9.48	9.48	9.48
FSL S11-1132	15	1.66	1.52	1.92	2.93	9.11	9.11	9.11
FSL S11-1290	15	1.66	1.42	1.92	2.84	9.37	9.37	9.37
FSL S11-1514	15	1.88	1.96	2.06	2.14	9.41	9.41	9.41
FSL S11-1613	15	1.77	1.66	1.94	2.01	9.23	9.23	9.23
FSL S11-1935	15	1.81	1.61	1.97	2.43	9.39	9.39	9.39
FSL S11-0890	27	1.8	6.86	7.81	7.81	7.81	7.81	7.81
FSL S11-1023	27	1.93	6.16	7.78	7.78	7.78	7.78	7.78
FSL S11-1132	27	1.52	6.93	7.41	7.41	7.41	7.41	7.41
FSL S11-1290	27	1.62	7.19	7.37	7.67	7.67	7.67	7.67
FSL S11-1514	27	1.92	7.71	7.71	7.71	7.71	7.71	7.71
FSL S11-1613	27	1.99	7.53	7.53	7.53	7.53	7.53	7.53
FSL S11-1935	27	1.82	5.6	7.69	7.69	7.69	7.69	7.69

Table 24. Trial 1 carnauba apple fruit surface coating solution *Listeria monocytogenes* log cfu/mL decrease

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
CFSAN023957	15	3.73	4.16	4.44	5.06	5.9	7	7
CFSAN073872	15	4.08	4.25	4.42	4.96	5.61	6.65	6.65
2010L-1723	15	4.09	4.19		4.91	5.2	6.2	6.2
2014L-6680	15	3.89	4.43	4.61	4.77	5.72	6.99	6.99
2014L-6695	15		5.81	5.41	5.41	7.41	7.41	7.41
4b1	15		4.72	4.76	5.4	7.15	8.1	8.1
H7858	15	3.8	4.58	4.89	5.82	6.71	8.42	8.42
CFSAN023957	27	3.94	5.12	5.8	7.4	7.4	8.4	8.4
CFSAN073872	27	3.89	4.79	5.38	7.42	7.42	8.42	8.42
2010L-1723	27	4.11	4.42	5.67	7.45	7.45	8.45	8.45
2014L-6680	27	3.94	4.99		7.47	7.47	8.47	8.47
2014L-6695	27	4.41	5.41	5.41	6.41	6.41	7.41	7.41
4b1	27	5.1	6.1	6.1	7.1	7.1	8.1	8.1
H7858	27	3.96	6.12	6.42	7.42	7.42	8.42	8.42

Table 24 (cont'd).

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
FSL S11-0890	15	4.63	4.58	4.94	5.26	6.13	7.25	8.5
FSL S11-1023	15	4.24	4.59	4.86	5.56	6.51	8.2	8.5
FSL S11-1132	15	4.16	4.87	5.18	5.78	7.12	7.5	8.5
FSL S11-1290	15	4.14	4.9	5.23	5.56	6.69	7.6	8.6
FSL S11-1514	15	4.14	4.72	5.02	5.51	6.36	7.5	8.5
FSL S11-1613	15	4.7	4.62	5.08	5.16	5.48	6.69	8.4
FSL S11-1935	15	4.1	4.67	4.91	5.31	6.3	7.39	8.5
FSL S11-0890	27	4.92	6.52	8.5	8.5	8.5	8.5	8.5
FSL S11-1023	27	4.76	6.71	7.9	8.5	8.5	8.5	8.5
FSL S11-1132	27	4.06	6.8	8.5	8.5	8.5	8.5	8.5
FSL S11-1290	27	4.64	6.17	7.3	8.6	8.6	8.6	8.6
FSL S11-1514	27	4.7	6.41	8.5	8.5	8.5	8.5	8.5
FSL S11-1613	27	4.62	6.38	8.4	8.4	8.4	8.4	8.4
FSL S11-1935	27	4.47	6.03	8.5	8.5	8.5	8.5	8.5

Table 25. Trial 2 carnauba apple fruit surface coating solution *Listeria monocytogenes* log cfu/mL decrease

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
CFSAN023957	15	3.1	4.43	4.7	5.17	6	6.99	7.1
CFSAN073872	15	3.78	4.27	4.36	4.83	5.65	6.74	7.72
2010L-1723	15	3.92	4.97	5.37	6.11	7.41	8.15	7.15
2014L-6680	15	3.22	4.75	5.31	6.01	7.87	8.47	7.47
2014L-6695	15	4.15	4.98	5.26	5.5	6.11	7.41	6.41
4b1	15	4.92	6.78	7.06	8.1	8.1	8.1	8.1
H7858	15	3.36	5.71	6.44	7.02	8.12	7.12	7.42
CFSAN023957	27	3.3	5.21	6.6	8.4	8.4	8.4	8.4
CFSAN073872	27	3.61	4.66	5.66	8.42	8.42	8.42	8.42
2010L-1723	27	3.54	7.75	8.45	8.45	8.45	8.45	8.45
2014L-6680	27	3.31		8.47	8.47	8.47	8.47	8.47
2014L-6695	27		7.41	7.41	7.41	7.41	7.41	7.41
4b1	27	4.79	8.1	8.1	8.1	8.1	8.1	8.1
H7858	27	3.51	7.47	8.42	8.42	8.42	8.42	8.42

Table 25 (cont'd).

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
FSL S11-0890	15	3.58	5.21	5.16	5.43	6.06	6.16	7.66
FSL S11-1023	15	2.44	5.89	6.67	6.37	6.97	6.72	7.67
FSL S11-1132	15	2.76	4.83	4.71	5.6	6.71	6.81	7.71
FSL S11-1290	15	2.94	4.95	5.16	5.92	7.55	7.67	8.15
FSL S11-1514	15	3.57	6.69	6.69	7.69	7.69	7.69	7.69
FSL S11-1613	15	3.89	5.86	5.8	7.64	7.64	7.64	7.64
FSL S11-1935	15	3.2	5.37	5.65	5.72	7.13	7.83	7.83
FSL S11-0890	27	4.33	6.32	6.76	7.66	7.66	7.66	7.66
FSL S11-1023	27	4.4	6.97	7.67	7.67	7.67	7.67	7.67
FSL S11-1132	27	3.04	6.81	7.01	7.71	7.71	7.71	7.71
FSL S11-1290	27	3.11	6.97	7.25	7.67	8.15	8.15	8.15
FSL S11-1514	27	4.76	7.69	7.69	7.69	7.69	7.69	7.69
FSL S11-1613	27	3.78	7.64	7.64	7.64	7.64	7.64	7.64
FSL S11-1935	27	3.54	7.23	7.83	7.83	7.83	7.83	7.83

Table 26. Trial 3 carnauba apple fruit surface coating solution *Listeria monocytogenes* log cfu/mL decrease

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
CFSAN023957	15	3.75	4.18	4.2	4.24	5.1	5.88	8.4
CFSAN073872	15	2.79	3.7	3.86	4.28	4.9	5.71	8.42
2010L-1723	15	4	3.96	4.05	4.24	4.65	5.05	8.45
2014L-6680	15	3.36	4.01	4.21	5.21	6.83	8.47	8.47
2014L-6695	15	2.16	2.99	3.14	3.48	4.23	5.59	7.41
4b1	15	3.73	4.19	4.76	5.02	7.32	8.1	6.78
H7858	15	3.53	4.39	4.88	5.17	6.28	7.58	8.42
CFSAN023957	27	3.67	3.57	5.36	8.4	8.4	8.4	8.4
CFSAN073872	27	2.96	4.68	5.64	7.94	8.42	8.42	8.42
2010L-1723	27	3.65	4.78	5.75	8.45	8.45	8.45	8.45
2014L-6680	27	3.53	4.56	5.52	7.99	8.47	8.47	8.47
2014L-6695	27	2.04	3.87	4.83	7.41	7.41	7.41	7.41
4b1	27	3.53	6.09	8.1	5.5	8.1	8.1	8.1
H7858	27	3.5	5.54	6.88	5.23	8.42	8.42	8.42

Table 26 (cont'd).

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
FSL S11-0890	15	4.06	3.83	3.95	3.93	9.51	9.51	9.51
FSL S11-1023	15	3.56	5.17	5.44	5.7	9.48	9.48	9.48
FSL S11-1132	15	3.88	5.3	6.41	6.11	9.11	9.11	9.11
FSL S11-1290	15	3.31	3.77	4.23	4.65	9.37	9.37	9.37
FSL S11-1514	15	4.76	5.67	6.41	6.41	9.41	9.41	9.41
FSL S11-1613	15	4.42	4.51	4.56	4.82	9.23	9.23	9.23
FSL S11-1935	15	3.15	4.68	5.04	5.69	9.39	9.39	9.39
FSL S11-0890	27	3.99	5.37	7.81	7.81	7.81	7.81	7.81
FSL S11-1023	27	3.61	7	7.78	7.78	7.78	7.78	7.78
FSL S11-1132	27	3.65	7.41	7.41	7.41	7.41	7.41	7.41
FSL S11-1290	27	3.03	4.89	5.77	7.67	7.67	7.67	7.67
FSL S11-1514	27	5.37	6.67	7.71	7.71	7.71	7.71	7.71
FSL S11-1613	27	5.04	6.83	7.53	7.53	7.53	7.53	7.53
FSL S11-1935	27	3.39	6.69	7.69	7.69	7.69	7.69	7.69

Table 27. Trial 1 morpholine apple fruit surface coating solution *Listeria monocytogenes* log cfu/mL decrease

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
CFSAN023957	15	1.91	2.63	3.61	4	4.5	6.66	6.66
CFSAN073872	15	1.83	2.46	2.99	2.96	4.37	5.17	5.17
2010L-1723	15	1.72	2.6	4.54	3.64	4.57	6.5	6.5
2014L-6680	15	1.96	2.71	3.38	4.19	4.66	7.15	7.15
2014L-6695	15	2.01	3.43	3.64	4.19	5.76	7.41	7.41
4b1	15	2.02	3.82		5.58	8.1	8.1	8.1
H7858	15	1.9	3.23	4.42	4.17	4.7	7.82	7.82
CFSAN023957	27	1.81	3.49	4.57	7.4	7.4	8.4	8.4
CFSAN073872	27	1.75	3.53	4.38	7.42	7.42	8.42	8.42
2010L-1723	27	1.84	3.61	5.05	7.45	7.45	8.45	8.45
2014L-6680	27	1.82	3.69		7.47	7.47	8.47	8.47
2014L-6695	27	2.39	3.96	5.41	6.41	6.41	7.41	7.41
4b1	27	2.1		6.1	7.1	7.1	8.1	8.1
H7858	27	1.83	3.74	6.42	7.42	7.42	8.42	8.42

Table 27 (cont'd).

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
FSL S11-0890	15	2.09	3.1	3.7	3.44	4.69	6.35	8.5
FSL S11-1023	15	2.05	3.77	4.03	4.32	5.08	8.5	8.5
FSL S11-1132	15	0.99	3.69	3.59	3.63	4.87	8.5	8.5
FSL S11-1290	15	2.14	3.64	3.47	3.62	4.55	6.43	8.6
FSL S11-1514	15	1.97	3.41	3.44	3.59	4.13	6.25	8.5
FSL S11-1613	15	1.96	3.95	4.27	4.54	5.03	8.4	8.4
FSL S11-1935	15	1.87	3.23	3.67	3.2	3.96	4.93	7.5
FSL S11-0890	27	2.17	3.2	8.2	8.5	8.5	8.5	8.5
FSL S11-1023	27	2.01	4.4	6.21	7.41	8.5	8.5	8.5
FSL S11-1132	27	2.09	4.23	8.5	7.65	8.5	8.5	8.5
FSL S11-1290	27	2.04	4.38	6.32	7.47	8.6	8.6	8.6
FSL S11-1514	27	1.91	3.96	6.5	7.55	8.5	8.5	8.5
FSL S11-1613	27	1.94	4.65	8.4	7.16	8.4	8.4	8.4
FSL S11-1935	27	1.98	4.36	8.2	8.05	8.5	8.5	8.5

Table 28. Trial 2 morpholine apple fruit surface coating solution *Listeria monocytogenes* log cfu/mL decrease

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
CFSAN023957	15	2.01	2.69	2.89	3.03	4.04	4.87	8.4
CFSAN073872	15	2.1	3.01	2.77	2.84	3.94	4.5	7.72
2010L-1723	15	2.15		2.83	2.92	3.84	4.6	7.15
2014L-6680	15	2.09		3.08	3.15	4.28	5.38	8.47
2014L-6695	15	2.41	2.66	3.13	3.1	2.72	5.34	6.93
4b1	15	2.25	2.93	3.73	3.93	3.61	6.62	7.8
H7858	15	2.11	3.49	2.96	3.03	4.42	5	8.12
CFSAN023957	27	1.94	3	3.55	4.83	8.4	8.4	8.4
CFSAN073872	27	2.01	3.2		6.12	8.42	8.42	8.42
2010L-1723	27	2.17	3.85	4.65	6.89	8.45	8.15	8.45
2014L-6680	27	1.99	2.77	3.34	4.48	8.47	8.47	8.47
2014L-6695	27	0.25	3.23	5.41	7.41	7.41	7.41	7.41
4b1	27	2.27	4.21	5.35	8.1	8.1	7.8	8.1
H7858	27	2.12		4.58	6.94	8.42	8.42	8.42

Table 28 (cont'd).

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
FSL S11-0890	15	1.79	2.93	3.08	4.62	6.66	7.66	7.66
FSL S11-1023	15	2.1	2.35	2.4	3.26	4.43	6.39	7.67
FSL S11-1132	15	1.49	2.6	2.26	3.35	5.71	7.71	7.71
FSL S11-1290	15	2.1	2.8	2.77	3.56	5.55	8.15	8.15
FSL S11-1514	15	1.74	2.89	2.36	3.51	5.69	6.99	7.69
FSL S11-1613	15	1.89	4.16	3.38	4.53	5.34	7.34	7.64
FSL S11-1935	15	2.04	2.79	2.77	4.27	6.35	7.83	7.23
FSL S11-0890	27	1.77	5.1	7.66	7.66	7.66	7.66	7.66
FSL S11-1023	27	1.96	4.41	7.19	7.67	7.67	7.67	7.67
FSL S11-1132	27	1.45	3.2	4.67	5.51	7.71	7.71	7.71
FSL S11-1290	27	1.85	3.64	5.2	6.08	7.67	8.15	8.15
FSL S11-1514	27	1.65	3.69	5.12	7.69	7.69	7.69	7.69
FSL S11-1613	27	2.04	4.55	5.74	7.64	7.64	7.64	7.64
FSL S11-1935	27	1.95	4.52	7.83	7.83	7.83	7.83	7.83

Table 29. Trial 3 morpholine apple fruit surface coating solution *Listeria monocytogenes* log cfu/mL decrease

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
CFSAN023957	15	1.91	3.45	3.03	3.88	5.56	8.4	8.4
CFSAN073872	15	1.73	2.32	2.78	2.88	4.24	7.72	8.42
2010L-1723	15	1.96	3.18	3.13	3.61	5.7	8.45	8.45
2014L-6680	15	1.88	3.06	3.02	3.52	5.11	7.69	8.47
2014L-6695	15	0.81	1.33	1.72	2.07	3.3	5.09	7.41
4b1	15	2.01	3.19	3.45	4.4	6.16	8.1	8.1
H7858	15	2.03	2.04	3.01	2.72	7.22	7.47	8.42
CFSAN023957	27	1.86	3.84	4.97	8.4	8.4	8.4	8.4
CFSAN073872	27	1.84	2.97	4.43	8.42	8.42	8.42	8.42
2010L-1723	27	1.9	3.61	4.99	8.45	8.45	8.45	8.45
2014L-6680	27	1.96	3.91	5.54	8.47	8.47	8.47	8.47
2014L-6695	27	0.71	2.59	3.85	6.41	7.41	7.41	7.41
4b1	27	2.16	3.52	4.89	4.12	8.1	8.1	8.1
H7858	27	1.98	3.75	4.64	8.12	8.42	8.42	8.42

Table 29 (cont'd).

Strain	Temp (°C)	Time (d) 0	Time (d) 0.5	Time (d) 1	Time (d) 2	Time (d) 4	Time (d) 7	Time (d) 14
FSL S11-0890	15		2.29	3.37	3.54	9.51	9.51	9.51
FSL S11-1023	15	1.91	2.4	3.42	3.27	9.48	9.48	9.48
FSL S11-1132	15	1.61	2.46	2.55	3.72	9.11	9.11	9.11
FSL S11-1290	15	1.7	2.44	2.98	3.1	9.37	9.37	9.37
FSL S11-1514	15	1.88	2.09	3.73	3.9	9.41	9.41	9.41
FSL S11-1613	15	1.78	2.27	3.61	3.94	9.23	9.23	9.23
FSL S11-1935	15	1.65	2.09	2.77	2.8	9.39	9.39	9.39
FSL S11-0890	27	1.79	4.24	7.81	7.81	9.51	9.51	9.51
FSL S11-1023	27	1.88	3.77	7.78	7.78	9.48	9.48	9.48
FSL S11-1132	27	1.81	4.34	7.41	7.41	9.11	9.11	9.11
FSL S11-1290	27	1.57	3.17	4.67	6.72	9.37	9.37	9.37
FSL S11-1514	27	1.81	4.33	7.71	7.71	9.41	9.41	9.41
FSL S11-1613	27	1.78	4.14	7.23	7.53	9.23	9.23	9.23
FSL S11-1935	27	1.8	3.02	7.69	7.69	9.39	9.39	9.39

APPENDIX 5: APPLE FRUIT SURFACE COATING SOLUTIONS APPLIED TO MEMBRANE FILTERS *LISTERIA MONOCYTOGENES* LOG CFU/ML DECREASE

Table 30. Trial 1 apple fruit surface coating solutions applied to membrane filters *Listeria monocytogenes* log cfu/ml decrease

Treatment	Temp (°C)	Cocktail	Time (d) 0	Time (d) 7	Time (d) 14
Control	4	Environmental	1.44	2.95	2.72
Control	4	Outbreak	1.74	2.52	2.47
Control	20	Environmental	1.44	1.97	2.05
Control	20	Outbreak	1.74	2.13	2.25
Shellac	4	Environmental	2.49	4.26	7.3
Shellac	4	Outbreak	2.84	4.46	7
Shellac	20	Environmental	2.49	7.3	7.3
Shellac	20	Outbreak	2.84	7	7.3
Carnauba	4	Environmental	2.37	4.89	3.35
Carnauba	4	Outbreak	2.33	4.05	3.36
Carnauba	20	Environmental	2.37	5.31	7.3
Carnauba	20	Outbreak	2.33	5.06	6.52

Table 31. Trial 2 apple fruit surface coating solutions applied to membrane filters *Listeria monocytogenes* log cfu/ml decrease

Treatment	Temp (°C)	Cocktail	Time (d) 0	Time (d) 7	Time (d) 14
Control	4	Environmental	1.36	2.16	1.93
Control	4	Outbreak	1.32	2.01	1.68
Control	20	Environmental	1.36	1.98	1.89
Control	20	Outbreak	1.32	1.65	2.45
Shellac	4	Environmental	2.03	3.42	4.1
Shellac	4	Outbreak	2.19	3.57	5.73
Shellac	20	Environmental	2.03	4.96	7.3
Shellac	20	Outbreak	2.19	3.58	7.3
Carnauba	4	Environmental	2.17	2.9	3.35
Carnauba	4	Outbreak	2.15	2.96	3.31
Carnauba	20	Environmental	2.17	5.2	7.3
Carnauba	20	Outbreak	2.15	4.22	6.6

Table 32. Trial 3 apple fruit surface coating solutions applied to membrane filters *Listeria monocytogenes* log cfu/ml decrease

Treatment	Temp (°C)	Cocktail	Time (d) 0	Time (d) 7	Time (d) 14
Control	4	Environmental	1.23	0.38	1.57
Control	4	Outbreak	1.43	0.36	1.57
Control	20	Environmental	1.23	2.52	2.94
Control	20	Outbreak	1.43	1.76	3.28
Shellac	4	Environmental	2.87	7.3	7.3
Shellac	4	Outbreak	3.76	6.82	7.3
Shellac	20	Environmental	2.87	7.3	7.3
Shellac	20	Outbreak	3.76	7.3	7.3
Carnauba	4	Environmental	3.04	3.38	3.92
Carnauba	4	Outbreak	2.79	4.08	3.1
Carnauba	20	Environmental	3.04	4.83	7.3
Carnauba	20	Outbreak	2.79	6.7	7.3

APPENDIX 6: APPLE FRUIT SURFACE COATING SOLUTIONS APPLIED TO GALA APPLES *LISTERIA MONOCYTOGENES* LOG CFU/ML DECREASE

Table 33. Apple fruit surface coating solutions applied to gala apples *Listeria monocytogenes* log cfu/ml decrease

Treatment	Trial	Time (d) 0	Time (d) 14
Control	1	4.37	5.62
Shellac	1	5.06	9
Carnauba	1	4.57	9
Control	2	4.17	6.9
Shellac	2	4.91	9
Carnauba	2	4.66	9
Control	3	4.31	6.35
Shellac	3	5.49	8.7
Carnauba	3	4.29	8.4