THE INFLUENCE OF ENVIRONMENTAL MICROBIOTA IN THE CONTROL OF L. MONOCYTOGENES

A Thesis in

Food Science

by

Priscilla Sinclair

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The thesis of Priscilla Sinclair was reviewed and approved by the following:

Jasna Kovac  
Assistant Professor of Food Science  
Thesis Co-Advisor

Luke F. LaBorde  
Professor of Food Science  
Thesis Co-Advisor

Darrel Cockburn  
Assistant Professor of Food Science

Robert F. Roberts  
Professor of Food Science  
Head of the Department of Food Science
Abstract

Foodborne pathogens cause an estimated 128,000 hospitalizations and 3,000 deaths in the United States of America each year. *L. monocytogenes* is one of the leading causes of foodborne illness related deaths in the USA. Many *L. monocytogenes* outbreaks originate from food packing and/or processing environment (FDA, 2015, 2020, 2021; Pouillot et al., 2016), demonstrating a need for enhanced cleaning and sanitizing procedures. In part, *L. monocytogenes* is able to survive in the environment by biofilm formation as well as beneficial relationships with other microorganisms. Biofilms are difficult to remove once established and shield the microorganisms within them from lethal levels of sanitizers. Multiple studies have characterized the microbiome of various food packing and processing environments; however, it is unknown if the microbial families identified are universally found in food processing environments or unique to those included in the studies. In this thesis we aim to improve the understanding of the distribution of bacterial families found in food processing environment by determining the core microbiota of animal- and plant- based food processing facilities as well as the taxa that are differentially abundant among them. We also aim to assess the efficacy of two biocontrol lactic acid bacteria (LAB) strains against *L. monocytogenes* in a quasi-biofilm assay.

In order to assess the first aim, literature review was conducted to identify papers that characterized the microbiota in food processing or packing environments using Illumina 16s amplicon sequencing. The published data from the identified papers were then analyzed using Mothur v 1.44.2 and underwent downstream analysis to determine the core microbiota in animal- vs plant-based environments as well as the differentially abundant taxa between the two environments. In order to assess the efficacy of two LAB biocontrol strains to inhibit *L. monocytogenes* they were first tested against phylogenetically distinct *L. monocytogenes* isolates.
in a spot inoculation assay. The strains were then grown with *L. monocytogenes* in apple packinghouse microbiome samples for 3-, 5-, and 15-days in order to assay their ability to inhibit *L. monocytogenes* in an attached biomass. The microbiota at experimental end point was characterized for all quasi-biofilms.

The secondary analysis included eight previously published studies that sampled food processing environments. The Families *Moraxellaceae* and *Flavobacteriaceae* were identified as the shared core microbiota between animal- and plant- based common core microbiota. The biocontrol LAB strains were able to inhibit all distinct *L. monocytogenes* isolates in a spot inoculation assay. However, the two biocontrol strains were unable to reduce *L. monocytogenes* in 3- and 5-day quasi-biofilms. The biocontrol strains were able to reduce *L. monocytogenes* in the 15-day quasi biofilms, however, all reductions were below 0.50 log MPN/mL. These studies will help to inform development of enhanced cleaning and sanitizing procedures for control of *L. monocytogenes*. 
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Chapter 1

Literature Review

1.1 Relevance of *Listeria monocytogenes* to Produce Safety

*Listeria monocytogenes* is a facultative anaerobe, Gram positive, rod shaped psychrotolerant bacterium. It can survive and grow in food postharvest and processing environments due to its ability to survive and grow at temperatures as low as 1°C (Azizoglu et al., 2009). It is also tolerant to desiccation (Kragh & Truelstrup Hansen, 2020; Hajime Takahashi et al., 2011; Truelstrup Hansen & Vogel, 2011; Zoz et al., 2017) and high salt concentrations (Pan et al. 2010). *L. monocytogenes* is a foodborne pathogen that is the cause of listeriosis in humans. Listeriosis is a foodborne infection that has a 24% mortality rate in those who contract the disease (Farber & Peterkin, 1991), resulting in estimate 1600 cases and 260 deaths a year (CDC, 2021; WHO, 2018). In the past decade there have been 21 multistate outbreaks associated with *L. monocytogenes* and many more recalls (CDC, 2021). Six of the twenty-one outbreaks were linked to contaminated produce, including cantaloupes (2011), bean sprouts and prepackaged caramel covered apples (2014), packaged salads and frozen vegetables (2016), and enoki mushrooms (2020). In the 2011 cantaloupe outbreak, there were 147 confirmed cases of listeriosis and 33 deaths. The outbreak investigation by the FDA detected the most closely related *L. monocytogenes* isolate to the outbreak strain in the packinghouse environment (FDA, 2011). Additionally, the investigation found that many of the packinghouse practices were conducive to *L. monocytogenes* survival and growth, such as the warm storage of fruit, standing water, and free water on the surface of the fruit after rinsing (FDA, 2011). In 2014, there was a multistate *L. monocytogenes* outbreak that was associated with caramel covered apples. This
outbreak resulted in 34 hospitalizations and 7 deaths, which resulted in four different companies recalling their products as they were the suspected source of the outbreak. The investigation of caramel apple outbreak found *L. monocytogenes* isolates closely related to the outbreak strain in a packinghouse that had not undergone a recall. The investigation concluded that the packinghouse was the source of contamination, which was then followed by a voluntary recall (FDA, 2015). This specific outbreak prompted an increase in the surveillance for *Listeria* spp. and *L. monocytogenes* in the tree fruit industry, resulting in an increase in recalls associated with apples in the next five years. There were recalls of fresh apple slices contaminated with *L. monocytogenes* in Michigan in 2017 (FDA, 2017b, 2017a), and in PA in 2015 (FDA, 2015). In 2017, fresh apple varieties were recalled for *L. monocytogenes* contamination by two different Michigan apple distributors. In a similar recall that occurred in 2019, a Michigan company requested that all fresh apple varieties be recalled due to potential *L. monocytogenes* contamination. Additionally, there was also a recall of peaches, nectarines, and plums that year (FDA, 2019). In October 2020, sliced fruit, including sliced apples was recalled for potential *L. monocytogenes* contamination (FDA, 2020). *L. monocytogenes* recalls and outbreaks result in major economic loss for not only individual packers and processors, but the whole industry. Extensive procedures are outlined in the reference guide and on the FDAs website detailing cleaning and sanitizing techniques to effectively control *L. monocytogenes*, these control procedures may not always be effective on *L. monocytogenes*. Therefore, there is a need for further research that assesses the efficacy of currently recommended cleaning and sanitizing techniques in inhibiting *L. monocytogenes* in different produce packing environments.
1.2 Prevalence of L. monocytogenes in produce processing/packing facilities

*L. monocytogenes* has been found in various produce processing or packing facilities due to its ability to survive and grow at low temperatures, survive desiccation, and its ability to form biofilms (Colagiorgi et al., 2017; Kadam et al., 2013; Lee et al., 2019). The survival of *L. monocytogenes* in the food processing environment presents an increased risk for contamination of produce where it may survive or even grow in certain conditions. For example, *L. monocytogenes* has been shown to survive on the surface of apples for more than 12 weeks of storage in cold rooms (Sheng et al., 2017). Due to the outbreaks and recalls caused by produce contaminated with *L. monocytogenes*, researchers have evaluated the presence of this pathogen in tree fruit processing and packing environments. The sampling studies tested non-food-contact surfaces for the presence of *L. monocytogenes*, as well as food-contact surfaces for the presence of *Listeria* spp. (Estrada et al., 2020; Ruiz-Llacsaahuanga et al., 2021; Tan et al., 2019) If one were to detect *L. monocytogenes* on a food contact surface it must be reported to the FDA, hence industry tends not to allow sampling of food contact surfaces for research purposes. Nevertheless, detection of *Listeria* spp. can be a good indicator of whether conditions in the sampled environment are conducive to *L. monocytogenes* survival and/or growth (UFPA, 2018).

In a 3-year study of 3 northeastern U.S. tree fruit facilities conducted in 2016-2019, the overall occurrence of *L. monocytogenes* was 17.5% with the highest occurrence in the packing line area (19.7%) followed by cold storage rooms (16.4%) and the packaging area (6.4%) (Simonetti, et. al, 2021). In a one-year study in 2017-2018 at the same packing facilities, *L. monocytogenes* was detected at zone 3 sites (underneath the packing line) in all facilities at varying frequencies, including one facility where it was detected in all samples taken (Tan et al., 2019). In another study that collected samples from 2018 to 2019, *Listeria* spp. were detected in
4.6% of tested samples in five commercial apple packinghouses that were sampled over a 2-year period (Ruiz-Llacsahuanga et al., 2021). This study included food contact surfaces and found that *Listeria* spp. were most frequently isolated from the wax coating area of the brush line, on polishing brushes, on the brushes underneath the fans in the dry area of the brush line, dryer rollers, and on stainless steel dividers (Ruiz-Llacsahuanga et al., 2021). This suggests that these areas on the packing line may be harborage sites of *Listeria* spp. in packinghouses. From July 2017 to March 2018, 11 produce packinghouses were sampled for *L. monocytogenes* in zones 2 and 4 (Estrada et al., 2020). Out of 1,588 collected samples, 3.2% were positive for just *L. monocytogenes*, 0.6% were positive for *L. monocytogenes* and *Listeria* spp., and 2.7% of samples were positive for just *Listeria* spp. The sampled sites that tested positive most often included cold storage rooms, drain sites, wet non-food contact surfaces, and areas outside the packinghouse (Estrada et al., 2020). The findings of Estrada et al. suggest that the travel from outside to within the facility may lead to *L. monocytogenes* contamination. Measures should be taken to remedy these issues. Furthermore, their findings demonstrate the need for enhanced sanitation and management of standing water in the facilities. In another study by Sullivan et al. (2020), three packinghouses and five fresh cut produce facilities were sampled over the course of a year for *L. monocytogenes* and *Listeria* spp., resulting in 2,014 collected sponge samples. The *L. monocytogenes* prevalence ranged from 0.8% to 5.8% in packing houses and 0.4% to 2.6% in the fresh-cut facilities; there was no facility with no positive samples (Sullivan & Wiedmann, 2020). The study focused on sampling sites it deemed difficult to clean, difficult to access and wet, as well as areas with build-up of organic debris, movable equipment, or high risk of contamination. There was no difference in prevalence of *L. monocytogenes* among sample sites (Sullivan & Wiedmann, 2020). In Ireland, *L. monocytogenes* was monitored in 48 food facilities.
in both food and environmental samples over the course of a year (Leong et al., 2014). Of the 48 facilities sampled, 62% had at least one positive *L. monocytogenes* sample. Six of the facilities sampled were produce processing facilities and in all six *L. monocytogenes* was detected in processing environment (Leong et al., 2014). *L. monocytogenes* was also detected in a mushroom slicing and packing facilities where 255 environmental samples were collected over 13 months (Murugesan et al., 2015). Of the 255 samples, 18.8% tested positive for *L. monocytogenes*. The authors identified the mushroom slicing area and a floor crack as the most likely contamination source, In another small mushroom processing facility, 184 samples were collected throughout the facility, including composting, tray filling line, growing rooms, and cooler room (Murugesan et al., 2015). *Listeria* spp. were detected in 15.8% of samples and *L. monocytogenes* in 1.6% of tested samples (Viswanath et al., 2013). Three studies found a high prevalence of *L. monocytogenes* in difficult-to-clean areas (Estrada et al., 2020; Ruiz-Llasahuanga et al., 2021; Sullivan & Wiedmann, 2020). Additionally, many of the studies sampled environments over time or sampled areas that were consistently positive for *Listeria* spp. and/or *L. monocytogenes*, suggesting possible persistence in these environments.

The prevalence and persistence of *L. monocytogenes* in food processing facilities is generally a concern, however it is an even greater concern when it is detected in facilities that produce ready-to-eat (RTE) products such as fresh produce. The prevalence of *L. monocytogenes* in many of produce processing or packing facilities demonstrates the importance of proper cleaning and sanitizing techniques that must be employed in order to control *L. monocytogenes*. The studies described above also highlight the importance and the role of difficult-to-clean/ hard-to-access areas in the persistence of *L. monocytogenes* in the produce processing or packing environment (Simonetti et al., 2021; Sullivan & Wiedmann, 2020; Tan et al., 2019). Many of the studies
(Chen et al.; Estrada et al., 2020; Leong et al., 2014; Murugesan et al., 2015) serotyped \textit{L. monocytogenes} isolated from collected samples. Isolates that were highly similar or clonal may represent a persistent \textit{L. monocytogenes} strain, while a higher diversity of \textit{L. monocytogenes} isolates collected over time may suggests that the \textit{L. monocytogenes} detected in the facility’s environment are transient and that \textit{Listeria} may be controlled effectively in these environments. In the above-described studies, the time and frequency of sanitation was not reported, which does not allow for the inference of relationships between cleaning and sanitizing practices and the prevalence of \textit{Listeria spp.} or \textit{L. monocytogenes}.

\subsection*{1.3 Biofilm Formation in Produce Facilities}

One of the contributing factors to the persistence of \textit{L. monocytogenes} is its ability to form biofilms both in monoculture as well as in multispecies cultures (Colagiorgi et al., 2017; Giaouris et al., 2013; Lee et al., 2019). Biofilms are consortia of microorganisms bound together by extracellular polymeric substances (EPS). EPS are biopolymers composed of polysaccharides, proteins, and DNA molecules produced by microorganisms (Flemming et al., 2007). EPS provide nutrients and a physical barrier that serves as a protection from environmental stressors (Jayathilake et al., 2017; Wolfaardt et al., 1999). The biofilm structure allows for microorganisms to stay in close proximity with each other, fueling cell to cell communication, competition, and growth (Flemming et al., 2007; Jayathilake et al., 2017).

The first step in biofilm formation is the reversible attachment to a surface (Tuson & Weibel, 2013). After a bacterium has adhered to a surface, it continues to grow into a monolayer and irreversibly attaches to the surface through van der Waals and hydrophobic interactions between the cell wall and the surface (Renner & Weibel, 2011; Tuson & Weibel, 2013). The microcolony
then starts to produce EPS and the microorganisms continue to grow into multilayered microcolonies (Flemming & Wingender, 2010). When biofilms mature, they often disperse the bacteria into the environment (Ma et al., 2009), which presents an increased food safety risk. There are multiple mechanism in which bacteria can attach and adhere to a surface, however most involve motility and surface attachment genes (Tuson & Weibel, 2013). *L. monocytogenes* can use flagella (Santos et al., 2019; O. Tresse et al., 2007; O. T. Tresse et al., 2009) as well as internalin genes and other genes that encode for secretion, transport, and signaling that enhance biofilm formation (Piercey et al., 2016; Popowska et al., 2017). Strains of *L. monocytogenes* that lack flagella have also been shown to form biofilm growth on static (dry) surfaces (Piercey et al., 2016). *L. monocytogenes* has been shown to form mature biofilms on food processing surfaces in 7 days at 30°C (Ripolles-Avila et al., 2018) at 37 °C, but not at 10 °C, after 24 hours (Lee et al., 2019). Strains of *L. monocytogenes* have also been shown to produce mature biofilms at 20 °C after 5 days of growth on stainless steel and polytetrafluoroethylene, as well as after 48 hours of growth at 18 and 25 °C in polystyrene 96-well plates (Chavant et al., 2004; Zameer et al., 2010). Surface hydrophobicity has an impact on the ability of *L. monocytogenes* to form biofilms (Bonaventura et al., 2008; Choi et al., 2013; H. Takahashi et al., 2010). One study that tested the ability of *L. monocytogenes* to grow biofilms on various food processing surfaces found that *L. monocytogenes* was capable of producing biofilms on stainless steel and concrete (Dygico et al., 2020). A study by Bonaventura et al. (2008) reported that temperature may have an effect on the hydrophobicity of the surface and *L. monocytogenes*, that resulted in increased surface adherence surfaces at higher temperatures (Bonaventura et al., 2008). However, this was not corroborated by other studies (Kadam et al., 2013; Santos et al., 2019). *L. monocytogenes* strains produced
greater amounts of biofilm growth in nutrient-depleted media that had salt levels from 0.85 to 7% (Kragh & Truelstrup Hansen, 2020; Lee et al., 2019; Pan et al., 2010).

$L.\ monocytogenes$ growth in biofilms in the environment is influenced not only by interactions with the physical environment, but also with the environmental microbiome. $L.\ monocytogenes$ biofilm growth can be inhibited or enhanced depending on the other species of bacteria present. The impact of $Pseudomonas$ spp. on $L.\ monocytogenes$ in biofilms and in planktonic form has been researched extensively (Buchanan & Bagi, 1999; Giaouris et al., 2013; Haddad et al., 2021; Maggio et al., 2021; Pang & Yuk, 2019; Puga et al., 2018). Some strains of $Pseudomonas$ spp. have been shown to deter (Alavi & Hansen, 2013; Al-Zeyara et al., 2011; Buchanan & Bagi, 1999; Farrag & Marth, 1989; Heir et al., 2018), while others were shown to enhance (Buzoleva et al., 2016; Haddad et al., 2021; Hassan et al., 2004) $L.\ monocytogenes$ planktonic and biofilm growth. This is typically due to competitive exclusion (Chorianopoulos et al., 2008; Leriche & Carpentier, 2000), which is defined as the elimination of a species from an environment when two different species have the same resource needs. $L.\ monocytogenes$ biofilm growth has also been shown to be deterred when grown with strains of $Staphylococcus$ spp., $Shewanella$ spp., $Acinetobacter$ spp., and $Serratia$ spp. (Alavi & Hansen, 2013; Heir et al., 2018; Rieu et al., 2008; Weiler et al., 2013). In contrast, $L.\ monocytogenes$ biofilm formation and growth is enhanced by strains of $Bacillus\ cereus$, $Lactobacillus\ plantarum$, and $Flavobacterium$ spp. (Alonso et al., 2020; Bremer et al., 2001; Haddad et al., 2021).

In previous studies that have reviewed the resident bacteria in food processing facilities, $Enterobacteriaceae$, $Acinetobacter$ spp., and $Pseudomonas$ spp. were reported to dominate the surfaces of food processing facilities (Møretrø & Langsrud, 2017). Resident bacteria are defined as the bacterial communities that persist in an environment over time (Møretrø & Langsrud,
In tree fruit packinghouses, the bacterial families that were found to be most prevalent include Flavobacteriaceae, Moraxellaceae, Weeksellaceae, Pseudomonadaceae, Burkholderiaceae, and Xanthomonadaceae (Tan et al., 2019). In a fresh-cut commercial processing facility, the most abundant species after sanitation were found to be Cupriavidus spp., Pseudomonas spp., Ralstonia spp., Stenotrophomonas spp., and an unknown species from the Enterobacteriaceae family (Gu et al., 2020). Additionally, a study that sampled fresh cut produce facility, reported P. fluorescens and Rahnella spp. as the most prevalent genera found among all (Liu et al., 2013). In dairy facilities, Enterococcus, Kocuria, Lactococcus, Acinetobacter, Streptococcus, Enterobacteriaceae, Debaryomyces, Pseudomonas, Psychrobacter, Stenotrophomonas, and Corynebacterium were reported as highly abundant in the processing environment (Anvarian et al., 2016; Bokulich & Mills, 2013; Johnson et al., 2021; McHugh et al., 2021; Stellato et al., 2015; B. Wang et al., 2019). Environmental sampling of meat-based facilities have found Enterobacteriaceae, Anaerobacillus, Bacillus, Leuconostoc, Streptococcus, Lactobacillus, Brochothrix, Clostridium, Yersinia, Staphylococcus, Escherichia, and Enterococcus in high relative abundance (Hultman et al., 2015; Schlegelová et al., 2010; Zwirzitz et al., 2020). Pseudomonas, Shewanella, and Photobacterium were the most commonly identified genera in fish processing facilities (Møretrø et al., 2016)

Many other bacteria have been isolated from food processing facilities that are capable of forming biofilms. These bacteria include Pseudomonas spp. (Araújo et al., 2016; Auerbach et al., 2000; Dynes et al., 2009; Hassan et al., 2004; Ma et al., 2009; Rossi et al., 2018; H. Wang et al., 2018), Acinetobacter (Cherif-Antar et al., 2016; Habimana et al., 2010; Simões et al., 2008), Moraxella spp. (Ely et al., 2019; Gunduz & Tuncel, 2006; Matejka et al., 2012), Flavobacteria spp. (Basson et al., 2008; Cai et al., 2013; Levipan & Avendaño-Herrera, 2017; Rios-Castillo et
al., 2018; Stewart et al., 2012), *Staphylococcus* spp. (Di Ciccio et al., 2015; Miao et al., 2017; Oniciuc et al., 2016; Planchon et al., 2006; Rode et al., 2007; Stepanović et al., 2003; Vázquez-Sánchez et al., 2013), lactic acid bacteria (Gómez et al., 2016; Habimana et al., 2011; Kubota et al., 2008; Muruzović et al., 2018; E. B. Somers et al., 2001; Tatsaporn & Kornkanok, 2020), *Xanthomonas* spp. (Dow et al., 2003; Sabuquillo & Cubero, 2021), *Enterobacteriaceae* (Allkja et al., 2020; Iversen et al., 2004; H. Wang et al., 2017) and *Bacillus* spp. (Chmielewski & Frank, 2003; Kwon et al., 2017; Morikawa, 2006). The presence of these bacterial genera and families in the food processing environment is a challenge for optimizing current cleaning and sanitizing techniques because biofilms are extremely difficult to remove. Biofilms support the growth of microorganisms and prevent microorganisms from being exposed to lethal levels of sanitizer (Chmielewski & Frank, 2003).

It is unknown what bacteria, if any, are common to all food processing and packing environments. Having knowledge of the universal microbiota of food processing and packing facilities would allow the development of cleaning and sanitizing treatments and routines to specifically eliminate not only *L. monocytogenes*, but bacteria that may facilitate the persistence of *L. monocytogenes* in the environment. For example, sanitizers benzalkonium chloride and peracetic acid are more effective in inhibiting gram positive bacteria (Fazlara & Ekhtelat, 2012; Ghotaslou & Bahrami, 2012) and ortho-phthalaldehyde is more effective on gram negative bacteria (Bridier et al., 2011). Additionally, disinfectants have been developed in order to dissolve biofilm structures (Aryal & Muriana, 2019), however they are not part of daily recommended sanitation schedules for *L. monocytogenes* control (FDA, 2020; UFPA, 2018). Identification of the microbiota in food processing environments have been attempted through microbiota analysis using next-generation sequencing. However, many of studies used this type
of sequencing, sampled only a small number of facilities. The question that remains unanswered is whether the identified microbiota in one studied facility is representative of all facilities of a similar type (e.g., plant- or animal-based food processing facilities). Comparative analyses of published data, including identification of core microbiota and differentially abundant microbiota in food processing environments could help to inform what bacterial families are universally present across food processing environments, and in specific types of food processing facilities.

1.4 The Use of Next-Generation Sequencing for Microbiota Characterization

Next-generation sequencing (NGS) is massive parallel sequencing in which millions of DNA fragments can be sequenced at the same time. Up until the development of NGS techniques, our ability to determine the composition of the food processing environment microbiota relied on isolating only culturable microorganisms, leaving out viable but non-culturable cells in addition to those below the detection limit (Hugenholtz et al., 1998). NGS characterizes genetic material in order to achieve a more complete understanding of the microbial composition of desired samples (Biddle et al., 2008; Riesenfeld et al., 2004). Microbiota can be characterized through amplicon sequencing where a gene marker present in all bacteria or all fungi is PCR-amplified and amplicons are sequenced. The amplification of conserved yet variable gene regions are imperative in identifying microorganisms in metagenomic sequencing (Shokralla et al., 2012). Different regions of the 16S rRNA gene are commonly used to identify different bacteria in samples (Janda & Abbott, 2007). The ITS gene, however, is often used for the identification of different fungi (Schoch et al., 2012).

Illumina MiSeq is commonly used for microbiota sequencing and is often preferred over Ion Torrent sequencing due to its greater sequencing depth and breadth of coverage (Frey et al.,
Additionally, Ion Torrent PGM was shown to have a larger error rate when compared to Illumina sequencing at 16S microbiota sequencing (Salipante et al., 2014). However, their ability to detect changes in genes is comparable when using different aligners (Lahens et al., 2017; Marine et al., 2020). Other technologies such as Nanopore and PacBio differ from Illumina in that they are long read sequencing technologies (Cui et al., 2020). Long read sequencing can increase taxonomic resolution, however the error rate for assembly is relatively high (Lang et al., 2020). Illumina sequencing uses a sequence by synthesis method with bridge amplification on the surface of a flow cell and the sequencing is then followed by quality filtering to eliminate low quality reads (Shokralla et al., 2012). Amplicon sequencing steps are the same as those followed for amplicon sequencing: DNA extraction, amplification of the target gene, attachment of adaptors for barcoding and library preparation, sequencing by synthesis for illumina followed by quality trimming, contig assembly and analysis (Caporaso et al., 2012). After sequencing, taxonomy is assigned and closely related sequences are clustered together into operational taxonomic units (OTUs), which can then be used for downstream analysis in order to determine taxonomic composition of microbiota. There are multiple software programs that perform this analysis, in this thesis, the software Mothur v. 1.44.2 is used for taxonomy and OTU assignment (Schloss et al., 2009).

Microbiota sequencing has allowed researchers to determine the microbial composition of samples by extracting the DNA and subjecting it to 16S rRNA amplicon sequencing. However, the technologies available today have an arbitrary total maximum number of sequences that instruments can generate, which results in compositional microbiome data. The results therefore do not account for every bacterium in the sample and also do not provide information about the absolute abundance of individual microorganisms in a sample (G. B. Gloor
et al., 2017). However, the microbiome sequencing and sequence analyses method that take the compositional nature of the microbiota sequencing data into account, allow researchers to study the composition of microbiota at a greater precision compared to using just culturing methods. Microbiome sequencing and sequence analyses methods therefore provide a more complete picture of the microbiota in studied environments (Riesenfeld et al., 2004). Although multiple studies have attempted to characterize the microbiota of food packing and processing environments, none have attempted to compare the microbiota composition using published data from multiple studies to identify the common bacterial families present across food packing and processing environments. Determining the core microbiota of all food processing environments as well as those specific to plant vs. animal product-based facilities, can help to inform cleaning and sanitizing practices in the industry, focusing on developing technologies that eliminate microorganisms that can adhere to surfaces and form biofilms that survive desiccation and sanitizer treatments. There are review papers that report on the bacterial families found in studies that sampled and sequenced food processing and packing environments. However, the difference in methodological procedures used to analyze sequences in each study may not be comparable.

1.5 Core Microbiome

The identification of the core microbiome in different ecological systems has become increasingly popular with the rise of next-generation sequencing techniques (Ainsworth et al., 2015; Cao et al., 2017; Gregorio et al., 2017; Wirth et al., 2018; Zaura et al., 2009). The core microbiome is defined as the taxa that are common to all or most microbial samples associated with an environment (Hamady & Knight, 2009; Shade & Handelsman, 2012; Turnbaugh et al., 2007). The common core microbiome identifies the most widespread taxa when looking at a
widespread environmental sample common to a host environment. The threshold for taxa identification in the common core of an environment ranges from 30 - 97% presence, in the literature (Ainsworth et al., 2015; Huse et al., 2012; K. Li et al., 2013). These thresholds have minimal explanation and are largely arbitrary. However, identifying the common core taxa is integral to gaining further insights into the interactions and function of the whole microbiome because commonly occurring organisms in an environment are most likely critical to the function of the microbiome (Shade & Handelsman, 2012). The members of the common core also give insight into the competitive nature of the bacteria present (Bauer et al., 2018; Hibbing et al., 2010), common introduction pathways, and the favorable growth conditions of the core taxa identified. Shared/membership cores are defined as the shared core microbiota within multiple environments.

In this thesis, the shared core are the bacterial families that are found in the core of plant and animal- based taxa. Determining the shared core further selects the most prevalent taxa. The attempt to determine the core microbiota of food post-harvest facilities in general and within facilities has not been widely explored in the past. Previous studies have sought to determine the microbiota in their studied facilities but have not explored the core microbiota in their analysis. This gap could potentially help to inform the development of enhanced procedures to control foodborne pathogens like \textit{L. monocytogenes}, as well as spoilage bacteria.

\textbf{1.6 Identifying Differentially Abundant Microbial Taxa}

Differential abundance testing aims to determine significant differences in microbial taxa among multiple ecosystems or environmental conditions. A differentially abundant taxon is a taxon that’s mean abundance is significantly different between two environments (Banerjee et
There are different differential abundance tests, but none have been regarded as the “best method” for performing differential abundance testing in the rapidly evolving field of microbiome data analyses (Hawinkel et al., 2019; Lin & Peddada, 2020; Wallen, 2021; Weiss et al., 2017). Two differential abundance methods, ANCOM (Mandal et al., 2015) and ALDEx2 (Fernandes et al., 2013), are commonly used and they account for the compositional nature of the data. ALDEx2 tests the null hypothesis that each taxon is not differentially abundant between the two conditions (Fernandes et al., 2013, 2014). ALDEx2 uses a Bayesian method to determine differentially abundant taxa: ALDEx2 first uses Monte Carlo sampling of Dirichlet distributions and is by default repeated 128 times to transform observational abundances to relative abundances. Relative abundance values are then transformed using centered log ratio (clr) (Fernandes et al., 2013). Both Welch’s t-test and Wilcoxon test are then performed on each taxon and the p-value is corrected using the Benjamini Hochberg correction (Fernandes et al., 2013). In addition to p-value, the effect size and overlap scores are calculated by taking the mean ratio of the difference of each feature between two groups and then divided by the maximum feature difference within groups (G. B. Gloor et al., 2016; Xia et al., 2018). Overlap is the proportion of the effect size that overlaps zero (Fernandes et al., 2014). Without considering the effect size and overlap cutoffs, ALDEx2 has been shown to have FDR > 0.05 (Lin & Peddada, 2020). The effect size cutoffs ranging from 1 to 2, depending on the desired level of conservatism have been applied in past studies (Fernandes et al., 2014; G. Gloor, 2018; Xia et al., 2018). With the effect size cutoffs recommended in Fernandes et al., 2014, ALDEx2 has been shown to have a very low number of false positives (Fernandes et al., 2014; G. Gloor, 2018). However, this comes at a price of a potentially excessive false negative rate.
ANCOM analysis is based on additive log ratio transformation (alr), this method has two assumptions: the mean log absolute abundance of two taxa is not different and the mean absolute abundance of all m taxa does not differ by the same amount between the two conditions (Mandal et al., 2015). ANCOM executes all available differential analyses, sequentially using each taxon as a reference taxon (Mandal et al., 2015). ANCOM adds a constant of 0.001 to all zeros before additive log ratio (alr) transformation (Mandal et al., 2015). The use of ANCOM-II for zero replacement is appropriate because it controls the false discovery rate with high power because it accounts for different types of zeros (Kaul et al., 2017). ANCOM-II treats sampling zeros by adding a small pseudo count, structural zeros by removing them and outlier zeros by missing at random and replaced by “NA” (Kaul et al., 2017). ANCOM uses an ANOVA like approach for significance testing and one can use either a standard t-test, Wilcoxon rank sums test, Mann-Whitney, permutation, or bootstrap resampling protocol to compute p-values (Mandal et al., 2015). This is then followed by Benjamini Hochberg correction. The number of rejections (null hypothesis) is then counted \( (W_i) \). A W cutoff of 0.70 percentile of the W distribution values is recommended by the authors of the method (Kaul et al., 2017), however W distribution percentile cutoffs range from 0.60 to 0.90, in order to achieve an FDR < 0.05. Both ALDEx2 and ANCOM have been shown to be competitive when compared to other differential analysis methods and are therefore used in this thesis (Fernandes et al., 2014b; Hawinkel et al., 2019; Lin & Peddada, 2020; Quinn et al., 2018; Wallen, 2021; Weiss et al., 2017).

1.7 Control of L. monocytogenes in Produce Processing/packing Environments

Industry guideline for the effective control of L. monocytogenes involves not only standard sanitation operating procedures (SSOPs), but also environmental monitoring (UFPA,
In order to properly develop and implement SSOPs, the risk of *L. monocytogenes* contamination and spread must be considered. This risk assessment includes taking into account the facility/ equipment design (Is it difficult-to-clean? Can it be disassembled?), water system (Where is the water coming from? How is it treated? Where does it go after leaving the facility?), packing bin storage and cleaning (Are the bins being stored outside? Are the bins being stored by standing water? Where in the facility do they go?), separation of wet and dry areas, traffic flow (Are outside materials being cleaned before they progress throughout the facility? How many items go from inside to outside?), air flow and the outer facility (Are there pests? Is there sanding puddles of water?) and how that may lead to the introduction and survival of *L. monocytogenes* in the facility (UFPA, 2018). Monitoring programs are recommended to ensure that the SSOPs in a facility are effective, detecting entrenched *Listeria* spp. before they contaminates the product, and determining necessary corrective action and problem areas (UFPA, 2018). Testing multiple spots around the packing line for *Listeria* spp. is advised, however, it is also advised to treat its detection as an indication that *L. monocytogenes* could survive in the environment and taking the proper corrective action as if it were the pathogen. Repeated detection of *Listeria* spp. in a sample location indicates persistence of *Listeria* spp. and corrective actions, including additional sampling to determine the contamination is originating from is advised and must be taken.

Cleaning and sanitizing SSOPs should include dry cleaning, pre-rinse, application of a foam cleaner and subsequent scrubbing of equipment, followed by post rinse, removal of condensation, standing water and inspection (FDA, 2020; UFPA, 2018). Finally, sanitizer should be applied to all equipment, walls, and floors. It is recommended that difficult-to-clean equipment be disassembled for cleaning and sanitizing (UFPA, 2018), however this is not an option for all facilities. Common sanitizers approved by the EPA used for the control of *L.*
*Listeria monocytogenes* include quaternary ammonia compounds (QAC), peracetic acid (PAA), chlorine dioxide (ClO₂), hydrogen peroxide, ozone, alcohol, and iodophores (UFPA, 2018). PAA, ozone, alcohols, ClO₂, and hydrogen peroxide are all oxidizing agents (Bang et al., 2014; Gordon & Rosenblatt, 2005; Guzel-Seydim et al., 2004; Zoellner et al., 2018), QAC are surface active compounds (Rahn & Van Eseltine, 1947). Ozone and ClO₂ are commonly used to sanitize the dump tank water (Ait-Oubahou et al., 2019). QAC, PAA, and ClO₂ are three of the most common sanitizers used on food equipment because of their low cost and widespread availability. In the study by Luque-Sastre, et al. (2008), QACs have been shown to have a minimum inhibitory concentration of 0.0015%- 0.006% against *L. monocytogenes* in planktonic form (Luque-Sastre et al., 2018). However, the same study found that the QACs tested against *L. monocytogenes* and *L. welshimeri* biofilms, failed to significantly reduce the *Listeria* spp. (Luque-Sastre et al., 2018). Similarly, when the efficacy of various disinfectants was tested against single strain foodborne pathogen biofilms, including *L. monocytogenes*, it was shown to be the least effective, only reducing the biofilms by 1-2 logs in 2 hours (Aryal & Muriana, 2019). In a study done by Korany, et al. 2018, the effect of QAC, PAA, ClO₂, chlorine, and ozonated water were tested against *L. monocytogenes* biofilms, both with and without organic matter (Korany et al., 2018). All of the sanitizers were tested at sanitizer appropriate levels, overall the single strain biofilms were inhibited by PAA the most, however its ability to reduce *L. monocytogenes* in the biofilms with organic matter decreased by up to 1.2 logs (Korany et al., 2018). The paper Hua, et. al, also found PAA to be the most effective at inhibiting *L. monocytogenes* multi-strain biofilms, compared to QAC, ClO₂, and chlorine (Hua et al., 2019). Many commercial sanitizer ability to inhibit *L. monocytogenes* is thwarted by the presence of organic matter (Aarnisalo et al., 2000; Gram et al., 2007; Hua et al., 2019; Ibusquiza et al., 2011;
Korany et al., 2018; Kuda et al., 2008; Somers & Lee Wong, 2004). When sanitizers are applied to dirty surfaces they are unable to destroy *L. monocytogenes*, because the organic matter increases its tolerance to the pathogen by preventing the sanitizer lethal contact (Dynes et al., 2009; Gram et al., 2007). Although, cleaning procedures may include scrubbing and the use of detergent, many surfaces may be difficult to clean due to placement in the facility, unhygienically designed equipment, which may lead to biofilm formation (Lee Wong, 1998; UFPA, 2018). If a bacterium has the ability to initiate biofilm formation, prolonged exposure to a surface may result in biofilm formation (Chmielewski & Frank, 2003; Lee Wong, 1998). The surface a biofilm is formed on may also impact sanitizer tolerance, (Bonaventura et al., 2008; Chaturongkasumrit et al., 2011; Hua et al., 2019; Park & Kang, 2017). These studies found that QACs and chlorine efficiency impact by surface type more than PAA. Coarse surfaces were found to have more tolerance to QAC, chlorine and ClO₂, which may be due to two reasons: i) there are more grooves and cracks that may act as harborage zones away from the sanitizer (Chaturongkasumrit et al., 2011; Hua et al., 2019) (ii) surfaces with increased roughness can support mature biofilm formation, with increased EPS, reduced contact with sanitizer (Chaturongkasumrit et al., 2011; Dygico et al., 2020). Biofilms are extremely difficult to remove from a surface, the bacteria are irreversibly bound to the surface and the EPS is able to act as a shield against cleaning and sanitizing agents as well as stick to the surface. Recommendations for biofilms removal typically involve scrubbing, the use of enzymes or “new generation” disinfectants that dissolve the structure, such as Sterilex Ultra solution, and Decon7 solution (Aryal & Muriana, 2019; UFPA, 2018).

The difficulty of biofilm removal from the food facility surfaces has led to research into the use of biocontrol strains to control pathogenic bacteria in biofilms. Biocontrols are
microorganisms that are able to destroy or thwart pathogens or other target bacteria. Biocontrols are able to inhibit target pathogens through infection followed by cell lysis (bacteriophages, competitive exclusion or the production of secondary metabolites (Alaniz Zanon et al., 2013; Bainton et al., 2004; Castellano et al., 2017; Gálvez et al., 2007; Ishaq et al., 2020; Mahony et al., 2011; Muriana, 1996; O’Flynn et al., 2004; Olanya et al., 2016; Perez et al., 2014). The use of lactic acid bacteria (LAB) strains have been shown to reduce foodborne pathogens and preserve food quality through competitive exclusion and the production of bacteriocins, the most commonly researched being nisin (Bolocan et al., 2017; Castellano et al., 2017; Linares-Morales et al., 2018; Muriana, 1996; Pei et al., 2020; Perez et al., 2014; Yin et al., 2020). The successful use of LAB as biocontrol strains against *L. monocytogenes* has been observed in in-vitro biofilms (Berrios-Rodriguez et al., 2020; Castellano et al., 2017; Rodriguez-López et al., 2018; Yin et al., 2020; Zhao et al., 2013).

There are three major classes of bacteriocins, class Ia and class IIa bacteriocins are able to inhibit *L. monocytogenes* (Kumariya et al., 2019). However, the ability of lactic acid bacteria to produce bacteriocins depends on quorum sensing, which is dependent on cell density (Kareb & Aïder, 2020). Strains of lactic acid bacteria are able to produce class I, II, and III bacteriocins (Zacharof & Lovitt, 2012). The most commonly used bacteriocins in the food industry is nisin, which can be produced by multiple lactic acid bacteria strains (Davies et al., 1997; Field et al., 2015; Fusieger et al., 2020; Ruiz et al., 2010; Siroli et al., 2016). The use of two lactic acid bacteria biocontrol strains, *Enterococcus durans* 152 and *Lactococcus lactis* subsp. lactis C-1-152 have also been tested against *Listeria* spp. in two poultry processing facilities, exhibiting up to 4.1 log CFU/mL reductions of *Listeria* spp. after repeated application (Zhao et al., 2006, 2013). The researchers concluded that the inhibition was due to competitive exclusion. However
in a follow up study, bacteriocins were successfully isolated from Enterococcus durans 152 (Du et al., 2017).

These studies have shown the commercial potential of using biocontrol strains biofilms to reduce Listeria spp. However, competitive interactions between bacteria in the environment may prevent the ability of biocontrols to reduce the target pathogen. In order to determine if these two biocontrol strains will be able control L. monocytogenes in the tree fruit packinghouse environment the biocontrols must be tested against L. monocytogenes when grown together in biofilms with the tree fruit packinghouse microbiome.

1.8 Purpose and Significance

L. monocytogenes most often causes disease in immunosuppressed individuals, making its presence in ready-to-eat facilities potentially hazardous to vulnerable people (WHO, 2018). The pathogen’s presence in packinghouses could lead to product contamination that could result in an outbreak or recall. In 2018, foodborne illness in the USA had an economic cost of 17.6 billion dollars a year, 18% of that cost due to L. monocytogenes related illness (Hoffmann & Ahn, 2021). An outbreak or recall could also hurt the entire industry as consumers may avoid the type of product altogether while shopping. Enhancing the control of L. monocytogenes in food packing and processing facilities could lead to less L. monocytogenes-related recalls and illness.

The ability of L. monocytogenes to form biofilms in food processing facilities has made the pathogen difficult to control and has led to its persistence in produce packing facilities as discussed above. Knowing the bacterial families in food processing environments can better prepare facilities for developing SSOPs to target bacteria that form biofilms and have beneficial relationships with pathogens. The resolution of bacteria that are common to all food packing and
processing environments may help to advance the cleaning and sanitizing agents and procedures recommended by industry. To date, there has not been a comprehensive comparative analysis of microbiota identified by next generation sequencing, which may unlock insights into the bacterial families that are common to food processing environments.

Previous research has looked at the potential use of LAB biocontrol strains to control *L. monocytogenes* and *Listeria* spp. *in vitro* and in poultry processing drains. The use of biocontrol to inhibit pathogens in biofilms may provide a solution for facilities that struggle to remove biofilms from their facility. However, the resident microflora that exists in different packing and processing environments may influence the ability of the biocontrol strains to inhibit the target pathogen. In order to determine if the biocontrol strains would be effective in tree fruit packinghouses, we must assess the efficacy of the biocontrol strains against *L. monocytogenes* in the presence of the native microflora in tree fruit packinghouses. The results of this research could help to control *L. monocytogenes* in biofilms that are difficult to remove from the environment in tree fruit packinghouses.

**1.9 Hypothesis and Objectives**

I hypothesize that the microbiota composition of animal- and plant- based environments will be different, but there will be a common core of bacterial families shared by both environments. I also hypothesize that the addition of *Enterococcus durans* 152 and *Lactococcus lactis* subsp. lactis C-1-152 to apple packinghouse microbiome samples will reduce *L. monocytogenes* in lab grown quasi-biofilms.

To test these hypotheses, I propose to:
1. Perform a comparative analysis of published data reporting microbiota composition in food processing environments to determine the common core microbiota in animal- and plant-based food processing environments.

2. Evaluate whether adding *Enterococcus durans* 152 and/or *Lactococcus lactis* subsp. lactis C-1-152 into apple packinghouse environmental microbiome can inhibit the growth of *L. monocytogenes* in a quasi-biofilm model.
Chapter 2

Comparative Analysis of Plant and Animal-Based Food Processing Environment Microbiota

2.1 Abstract

Foodborne pathogens cause an estimated 128,000 hospitalizations and 3,000 deaths in the U.S. each year. Many foodborne outbreaks originate from food packing and/or processing environments (FDA, 2015, 2020, 2021; Pouillot et al., 2016), demonstrating a need for enhanced foodborne pathogens controls. An estimated 30 to 40 percent of the food supply is wasted each year, this is in part due to spoilage, also demonstrating a need for enhanced spoilage organism controls. In part, these microorganisms able to survive in the environment by biofilm formation as well as beneficial relationships with other microorganisms. Multiple studies have characterized the microbiome of various food packing and processing environments; however, it is unknown if the microbial families identified are universally found in food processing environments or unique to the one.

Eight studies were selected by searching peer-reviewed publications published between 2015 and 2020 for studies that complied with inclusion criteria. The inclusion criteria were: (i) microbiota was sequenced using next generation sequencing, (ii) sequences were publicly available, (iii) samples were collected from built food processing environment. Identified studies were then further reviewed to exclude those that did not meet our inclusion and file formatting criteria: (i) a study that did not use Illumina sequencing (i.e., it used older sequencing technologies), (ii) a study that did not target 16S rRNA gene marker, or (iii) sequences linked with a study were not demultiplexed. All sequences were analyzed using Mothur v.1.44.2. The common core microbiota and shared common core microbiota between
plant- and animal-based environments were identified as bacterial families that were found in 50% or more of animal- or plant-based samples and in a 0.10% or greater relative abundance. The difference in microbiota composition between plant- and animal-based environments were evaluated by PERMANOVA and differential abundance analysis.

The bacterial families Moraxellaceae and Flavobacteriaceae were identified as the shared core of plant- and animal-based environments. The common core bacterial families found in plant-based environments were Moraxellaceae, Flavobacteriaceae, Pseudomonadaceae, Sphingomonadaceae, Xanthomonadaceae, and Comamonadaceae. The common core bacterial families found in animal-based environments were Moraxellaceae, Flavobacteriaceae, Staphylococcaceae, Rhodobacteraceae, Lachnospiraceae, Ruminococcaceae, and Enterobacteriaceae. The plant-and animal-based environments were different (p<0.01) according to PERMANOVA. Two differential abundance calculations were included, ALDEx2, which identified 3 bacterial families as differentially abundant and ANCOM, which identified 52 families as differentially abundant. In both plant- and animal-based environments the core bacterial families contained spoilage organisms and organisms with biofilm forming abilities. The results of this study will help to inform the development of cleaning and sanitizing enhancements designed to control both pathogens and spoilage organisms.

2.2 Introduction

Each year, there are an estimated 48 million cases of illness caused by foodborne pathogens, which result in estimated 128,000 hospitalizations and 3,000 deaths in the U.S. (CDC, 2018). In the past five years there have been seventy-three reported multi-state foodborne outbreaks in the United States of America (CDC, 2018). Many foodborne outbreaks can be
traced back to the food packing and/or processing environment as evidenced by several recent
recalls (FDA, 2015, 2020, 2021; Pouillot et al., 2016), demonstrating a need for procedural
enhancements in cleaning and sanitizing. Additionally, food spoilage bacteria contribute to food
waste, which is estimated to be between 30 and 40 percent of the food supply (USDA, 2021),
further calling for enhancements in cleaning and sanitizing. The Food and Drug Administration
(FDA) recommends cleaning and sanitizing routines for physical removal of debris and soils,
followed by the application of chemical cleaners to remove surface soils, and lastly the
application of a sanitizer to inactivate foodborne pathogens and food spoilage organisms (FDA,
2020). Commercial cleaning chemicals and sanitizers are formulated for use in many different
types of food processing facilities to remove soils and kill microorganisms. However, the
efficacy testing of sanitizers has emphasized reducing foodborne pathogens in the environment
(Aryal & Muriana, 2019; Cruz & Fletcher, 2012; Davidson et al., 2013; Ruiz-Cruz et al., 2007,
p.; Sinde & Carballo, 2000; Somers & Lee Wong, 2004; Taylor et al., 1999) Cleaning and
sanitizing recommendations are general in nature and may not be equally effective in all food
processing environments due to differences in the specific types of soils, equipment surfaces, and
environmental microbiota.

Bacteria that make up the residential microbiota of food processing facilities can be
introduced into the food processing environment and cross-contaminated through incoming raw
materials and food ingredients, equipment or tools, and employees (Fox et al., 2014; Hultman et
al., 2015; Leong et al., 2014; Møretrø & Langsrud, 2017; Schön et al., 2016). Conditions in the
food processing environment may facilitate the growth of microorganisms, especially in facilities
with high relative humidity, dripping condensate and standing pools of water, temperatures that
support bacterial growth, and an ample supply of nutrients (Carlez et al., 1994; Carpentier & Cerf, 2011; Heyndrickx, 2011; Lee Wong, 1998).

Cleaning and sanitizing practices play a major role in shaping the composition of environmental microbiota. Infrequent or improper application of cleaning and sanitizing chemicals can lead to the formation of biofilms. Many microorganisms are able to form and/or colonize biofilms which are characterized by the formation of extracellular polymeric substances (EPS) that can protect colonized pathogens and spoilage microorganisms by reducing the diffusion of cleaning and sanitizing chemicals into the biofilm structure (Wolfaardt et al., 1999). When pathogens like *L. monocytogenes* are incorporated into biofilm, they have been shown to exhibit tolerance to sanitizers such as quaternary ammonia compounds (QAC), chlorine dioxide (ClO2), benzalkonium chloride (BAC), and peroxides (Ibusquiza et al., 2011; Pan et al., 2006). Other bacteria common to the food environment such as, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, *Staphylococcus aureus* also exhibited tolerance to commonly used sanitizers when incorporated within biofilms (Abdallah et al., 2015; Lee et al., 2015; Huhu Wang et al., 2018).

Microbial interactions of microorganism within the food processing microbiome can also affect the survival and growth of environmental pathogens by affecting nutrient availability (Webb et al., 2002), formation of secondary metabolites (Shank & Kolter, 2009; Tyc et al., 2017; Yan et al., 2018), and the extent to which EPS is formed (Flemming et al., 2007; Flemming & Wingender, 2010; Wolfaardt et al., 1999). All these factors can affect the ability of bacteria to survive and grow in processing environments. Understanding the composition of the members of microbiota that are found broadly across food processing facilities (i.e., common core microbiota) is the first step towards studying the role of residential microbiota in pathogen
survival and persistence in food processing environments (Shade & Handelsman, 2012). Acquiring a greater understanding of the type of bacteria present in processing facilities could inform efforts to improve food industry cleaning and sanitizing practices. Specifically, by targeting microorganisms that are known to enhance the growth of foodborne pathogens and spoilage organisms as well as those that may enhance the survival of pathogens and spoilage organisms in the food processing environments.

Previous studies have reported a wide variety of bacterial families in food processing environments (Barría et al., 2020; Caraballo Guzmán et al., 2020; Dzieciol et al., 2016; Einson et al., 2018; Fagerlund et al., 2017; Falardeau et al., 2019; Gu et al., 2019; Guzzon et al., 2017; Hultman et al., 2015; Jarvis et al., 2018; Kamimura et al., 2020; Kang et al., 2019; Maes et al., 2019; Pothakos et al., 2015; Quijada et al., 2018; Rodríguez-López et al., 2019, 2020; Stellato et al., 2015; Tan et al., 2019; Wang et al., 2018; Zwirzitz et al., 2020) Each of these studies used different analytical methods elucidate the microbial diversity and ecology within processing environments. However, it is unknown if the microbiota identified in these studies is representative of the microbiota found across all food processing environments or if it is unique to the specific facilities studied. Comprehensive reviews of the scientific literature have been published on the resident bacteria found within food processing facilities (Chmielewski & Frank, 2003; Møretrø & Langsrud, 2017). The genera/families reported as most prevalent included Pseudomonas, Acinetobacter, Staphylococcus, Enterobacteriacea, as well as spore-forming and lactic acid bacteria (Chmielewski & Frank, 2003; Møretrø & Langsrud, 2017). These taxa all have spoilage potential, further outlining how the microbiomes in food processing facilities impact food quality and safety.
To improve the understanding of the distribution of bacterial families found in food processing environment, we conducted a comparative analysis of published microbiota data to identify microbial taxa that are present across all surveyed facilities and constitute a core microbiome of food processing facilities. We conducted the analyses using a compositional analysis framework that allows for detection of changes in microbiota composition in the absence of information depicting total microbial load within samples (G. B. Gloor et al., 2017). We aimed to determine the core microbiota of animal- and plant- based food processing facilities as well as the taxa that are differentially abundant among them. The results of this study could inform the development of improved strategies for controlling food spoilage and pathogenic bacteria.

2.3 Materials and Methods

2.3.1 Study Inclusion Criteria

The databases Web of Science, Google Scholar, and Pubmed were used to search for studies reporting the composition of microbiota in built food processing environment that were included in this study. Peer-reviewed publications published between 2015 and 2020 were searched for using the terms “environmental microbiome in food facility”, “environmental microbiome in packinghouse”, “microbiome found in meat processor”, “microbiome in food facility”, “microbiome in produce facility”, “microbiome in dairy facility”, “characterization of food facility microbiome, “characterization of packinghouse microbiome”, “food processing microbiome”. Papers with relevant titles were reviewed to determine whether they meet the criteria that allowed inclusion into our comparative analysis of built food processing
environmental microbiota: (i) microbiota was sequenced using next generation sequencing, (ii) sequences were publicly available, (iii) samples were collected from built food processing environment. Identified studies were then further reviewed to exclude those that did not meet our inclusion and file formatting criteria: (i) a study that did not use Illumina sequencing (i.e., it used older sequencing technologies), (ii) a study that did not target 16S rRNA gene marker, or (iii) sequences linked with a study were not demultiplexed.

2.3.2 Microbiota Analysis

The studies included in this comparative microbiota analysis amplified different regions of the 16S rRNA gene: V3 (Falardeau et al., 2019), V4 (Caraballo Guzmán et al., 2020; S. Kang et al., 2019; Tan et al., 2019) and V3-4 (Einson et al., 2018; Kamimura et al., 2020; X. Wang et al., 2018; Zwirzitz et al., 2020). Comparing taxonomic composition of microbiota assigned based on different regions of the 16S rRNA gene can create a bias. This bias may go all the way up to the phylum level, although the majority of differences are observed at genus level thus influencing abundance values (Albertsen et al., 2015; L. Cai et al., 2013; Drengenes et al., 2021; Poirier et al., 2018; Winand et al., 2019). To minimize genera bias, microbiota composition was compared at a family taxonomic level. Sequences were downloaded from the NCBI SRA database using SRA Toolkit v. 2.8.2. Sequences were analyzed using Mothur v. 1.44.2 (Schloss et al., 2009) following the MiSeq standard operating procedure (Kozich et al., 2013). First, contigs were created by generating the reverse complement of the reverse reads for each sample. The reads were then joined into contigs by aligning the pairs of sequences and identifying positions of disagreement. Contigs that were not within the 2.5% - 97.5% quartile of all assembled lengths as well as sequences with ambiguous bases were excluded from further
processing. The remaining sequences were then aligned to the Silva database v. 132. Sequences that aligned before or after the target site of alignment were discarded. The expected region of alignment varied depending on the region of the 16S rRNA gene that was amplified. The sequences were then de-noised and chimera sequences were removed. Taxonomy was then assigned using the 16S rRNA reference RDP v. 16 (Edgar, 2018). Sequences that were classified as archaea, eukaryotes, chloroplast, mitochondria, or were unclassified, were removed. Sequences that were 97% or more similar were assigned the same OTU using the OptiClust method. Finally, taxonomy was assigned to OTUs. The resulting OTU and taxonomy file, were combined using R v. 3.6.1 with the package Phyloseq v. 1.28.0 to produce a merged file with assigned taxonomy to OTU counts in each sample. The data was normalized after datasets were merged and study labels were assigned to each sample to account for differing sequencing depths. The data was not normalized before merging data sets because rarefaction (Sze & Schloss, 2016) results in the loss of important data, which can result in misleading conclusions (Gloor et al., 2017).

2.3.3 OTU Normalization and Principal Component Analysis

The OTU table with assigned taxonomy was first processed to replace 0 values using the count zero multiplicative method using R package zCompositions v. 1.3.4. (Xia et al., 2018), followed by conversion of counts to proportions. Finally, centered log transformation (CLR) was performed in order to transform the compositional data into real space in R v. 3.6.1 (Xia et al., 2018). Singular value decomposition was then used to carry out principal component analysis to evaluate whether samples cluster based on meat-, dairy or plant- based food processing.
environment origin. R packages vegan v. 2.5.7, compositions v. 2.0.0 and ggplot2 v. 3.3.3 were used to plot the first two principal components to visualize clustering.

2.3.4 Identification of Core Microbiota in Animal- or Plant-based Food Processing Facilities

In this study the common core microbiota of a plant- or animal-based environment was defined as the bacterial families that were found in 50% or more of animal- or plant-based samples and in a 0.10% or greater relative abundance in samples from plant- or animal-based food processing environment (Ainsworth et al., 2015; Philippot et al., 2013). In order to identify the core bacterial families, the R package Microbiome v. 1.6.0 was used and the script from L. Lahti and S. Shetty (Lahti & Shetty, 2017) was adapted.

2.3.5 PERMANOVA and Differential Abundance Calculation

PERMANOVA analysis was performed based on the calculated Aitchinson distances to test whether there are significant differences in the microbiota composition among plant, meat, and dairy based food processing environments. PERMANOVA analyses were carried out using the R package pairwiseAdonis v. 0.0.1

Differential abundance analysis was carried out to identify families that were present in significantly different relative abundances in plant- compared to animal-based food processing environments. Significant families were identified using two complementary differential abundance analyses methods: ANCOM (Mandal et al., 2015) and ALDEx2 (Fernandes et al., 2014; G. B. Gloor et al., 2016). These two methods were chosen because the tests use different statistical methods to perform different abundance testing. Both ALDEx2 and ANCOM determined differential abundance using log-transformed family counts. ALDEx2 analysis was
carried out using R package ALDEx2 v. 1.22.0 (Xia et al., 2018). ANCOM was performed using the pipeline ANCOM v 2.1 using R packages readr v. 1.4.0 (Wickham et al., 2020) and tidyverse v. 1.3.0. (R Core Team, 2019). ALDEx2 infers significance based on a p-value obtained from Welch’s T and Wilcoxon test, and Benjamini Hochberg correction for multiple comparisons. Unlike ALDEx2, ANCOM did not infer differentially abundant taxa by evaluating p-value, as it drew conclusions from the Mann-Whitney U test statistic. We considered families identified by ANCOM with FDR test statistic cutoff of 0.90 percentile of the W value distribution as significant. Furthermore, we considered families identified by ALDEx2 as having a Benjamini Hochberg corrected p-value <0.05 and an effect size > 1.5 as significant. We performed a centered log ratio transformation of the family count table and calculated the CLR mean difference (effect size) to visualize the results of ANCOM (S. Lee et al., 2019). The results of both the ANCOM and ALDEx2 analysis were visualized using R package qqplot2 v 3.3.3. The methods used in this analysis can be found at https://github.com/pcs5238/companal.

Results

2.4.1 Selection of Studies for Inclusion in Comparative Microbiota Analyses

Twenty-two studies were identified in the scientific peer-reviewed literature databases using the inclusion criteria outlined in methods (Barria et al., 2020; Caraballo Guzmán et al., 2020; Dzieciol et al., 2016; Einson et al., 2018; Fagerlund et al., 2017; Falardeau et al., 2019; Gu et al., 2019; Guzzon et al., 2017; Hultman et al., 2015; Jarvis et al., 2018; Kamimura et al., 2020; Kang et al., 2019; Maes et al., 2019; Pothakos et al., 2015; Quijada et al., 2018; Rodríguez-López et al., 2019, 2020; Stellato et al., 2015; Tan et al., 2019; Wang et al., 2018; Zwirzitz et al., 2020). Three studies were excluded on the basis of our exclusion criteria (Barria et al., 2020;
Fagerlund et al., 2017; Maes et al., 2019) due to sequencing of select colonies grown on agar media as oppose to amplifying 16S rRNA gene marker using DNA extracted from whole environmental samples. Additional studies were excluded due to the use of pyrosequencing (Dzieciol et al., 2016; Guzzon et al., 2017; Hultman et al., 2015; Pothakos et al., 2015; Stellato et al., 2015, Calasso et al., 2016), Ion Torrent sequencing (Rodríguez-López et al., 2019, 2020), or clone by clone sequencing (Quijada et al., 2018). Lastly, two studies were excluded due to unavailability of demultiplexed sequence files (Gu et al., 2019), and due to sampling only food and not the food processing environment (Jarvis et al., 2018). Finally, sequencing data from eight studies were included for comparative microbiota analysis in this study (Table 2.4.1). Table 2.4.1 lists’ references, amplification region, and food processing facility type (i.e., plant- or animal-based). All included studies targeted V3, V4 or V3-V4 region of the 16S rRNA gene (Table 2.4.1). To minimize taxonomic assignment bias due to different 16S rRNA variable region being sequenced in different studies, all data were analyzed at a family taxonomic level (García-López et al., 2020).
Table 2.4.1. Studies included in the comparative microbiota analysis

<table>
<thead>
<tr>
<th>Code</th>
<th>Paper</th>
<th>Category</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>S8</td>
<td>Tan, X. et al. The occurrence of <em>Listeria monocytogenes</em> is associated with built environment microbiota in three tree fruit processing facilities. Microbiome 7, 115 (2019).</td>
<td>Plant Based</td>
<td>V4</td>
</tr>
</tbody>
</table>

2.4.2 Clustering of Samples from Plant- and Animal-based Food Processing Facilities Based on Family Composition

Principle component analysis (PCA) was performed and the first two principal component (PCs) were plotted to visualize the overall differences in the microbiota composition among samples collected from plant-, dairy- and meat-based food processing environments. The first two PCs explained 45.32% of the variance in the compositional data: 33.36% by PC1 and 11.96% by PC 2. There was clear clustering of samples from meat- (Caraballo Guzmán et al.,
2020; S. Kang et al., 2019; Zwirzitz et al., 2020) or plant-based (Caraballo Guzmán et al., 2020; Einson et al., 2018; Tan et al., 2019; X. Wang et al., 2018) food processing environments based on PC 2. Within the group of samples collected from animal-based food processing facilities, samples collected from dairy-based environments (S2 and S6) appeared to have a distinct composition of microbiota compared to those collected from meat-based (Caraballo Guzmán et al., 2020; S. Kang et al., 2019; Zwirzitz et al., 2020) food processing facilities, based on clustering along the PC1 axis.

Samples from plant-based facilities (N=4; n=205) that processed liquor, pasta, tree fruit, and fermented vegetables appear to be more similar to each other than samples from animal-based facilities that processed dairy (N = 2; n = 364) and meat (N = 3; n = 397). Within the plant-, meat-, and dairy-based food processing facilities, samples from individual studies clustered together, suggesting a study effect. This study effect was confirmed as all studies’ microbiota compositions were found to be significantly different from each other using PERMANOVA analysis (Appendix B.3.1). There was clear clustering of samples by V3 vs. V3-V4 and V4, region of the 16S rRNA gene that was amplified. However, only one study amplified the V3 region, so it is unknown if the sample clustering was affected by the variable region or by facility type, study, or other factors in the PCA (Appendix B.3.2).
Figure 2.4.1. Clustering of samples based on PCA analysis of bacterial families. Samples are color-coded based on the study they originated from. Squares denote samples originating from plant-based facilities, triangles denote samples originating from dairy-based food processing facilities, and circles denote samples originating from meat-based food processing facilities.

2.4.3 Identification of Core Microbiota

We further investigated the shared common core microbiota across animal- and plant-based processing environments, as well as differences in the common core microbiota between plant-based and animal-based food processing facilities. The core microbial families shared between plant- and animal- based food processing environments were *Moraxellaceae*, *Flavobacteriaceae*, and Bacteria unclassified. The core bacterial families identified for animal-
based food processing environments were the shared core families Moraxellaceae, Flavobacteriaceae as well as Staphylococcaceae, Rhodobacteraceae, Lachnospiraceae, Ruminococcaceae, and Enterobacteriaceae. The rest of the identified core families from animal-based facilities were unidentified families from orders Firmicutes, Actinomycetales, Bacteroidetes, Clostridiales, and Clostrida. The core bacterial families identified in plant-based food processing environments were Moraxellaceae, Flavobacteriaceae as well as Pseudomonadaceae, Sphingomonadaceae, Xanthomonadaceae, and Comamonadaceae. Figure 2.4.2 shows the relative abundance of core families identified in animal- and plant-based food processing environments.

Figure 2.4.2. Relative Abundance of the Core Bacterial Families in Animal- and Plant-Based Food Processing Environments. The circle size represents the relative abundance of the core bacterial families detected in animal- and plant-based food processing environments. The largest
point represents 0.8% relative abundance, and the smallest point represents 0.2% relative abundance.

2.4.4 Differentially Abundant Families Found in Plant- and Animal-based Food Processing Environments

PERMANOVA analysis found that samples from plant- and animal- based studies were significantly different (p=0.001), differential abundance was performed to identify the significantly different bacterial families.

ALDEx2 identified 112 families as differentially abundant between plant- and animal- based food processing environments, based off of p-value (Figure 2.4.3). For the ALDEx2 analysis results, the families with an effect size > 1.5 were considered significant, 3 families passed this criterium: Ruminiconocaceae, and unidentified families from the Firmicutes and Clostridiales order. Based on the ANCOM analysis, using the recommended FDR cutoff of 0.90 percentile of the W distribution, 52 taxa were identified as differentially abundant between plant- and animal-based food processing facilities (Mandal et al., 2015).

The results of both ANCOM and ALDEx2 analyses are displayed in Figure 2.4.3 where the red bars represent the bacterial families that were significantly different amongst the two groups. All differentially abundant families identified by either of the two methods are listed in Table 2.4.2.

The families that were identified as the core microbiota for plant- or animal- based environments, but not a part of the shared core, were found to be differentially abundant based on the ANCOM analyses. However, the family’s presence in the core microbiota of an environment does not indicate that they have a significantly higher abundance in said environment. For example, the family Enterobacteriaceae was found to be significantly different
amongst the two groups and part of the core microbiota of animal processing facilities, but the
group had a higher abundance in the plant-based samples (0.3% vs 0.06%) it was present in
(Figure 2.4.2).

Table 2.4.2. Results from ALDEx2 and ANCOM analyses.

<table>
<thead>
<tr>
<th>Family</th>
<th>ALDEx2</th>
<th>ANCOM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaplasmataceae</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>Bacteria_unclassified</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>Bacteroidaceae</td>
<td>-</td>
<td>x</td>
</tr>
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<td>Bacteroidales_unclassified</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>Bacteroidetes_unclassified</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>Bartonellaceae</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>Brucellaceae</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>Campylobacteraceae</td>
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<td>x</td>
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<td>x</td>
</tr>
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<td>-</td>
<td>x</td>
</tr>
<tr>
<td>Clostridiales_unclassified</td>
<td>x</td>
<td>x</td>
</tr>
<tr>
<td>Comamonadaceae</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>Cytophagaceae</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>Dermabacteraceae</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>Dermacoccaceae</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>Desulfovibrionaceae</td>
<td>-</td>
<td>x</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>-</td>
<td>x</td>
</tr>
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<td>Xanthomonadaceae</td>
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Figure 2.4.3. Differentially abundant families identified in animal- and plant-based food processing environments using ALDEx2 (A) and ANCOM (B). The x-axis represents the effect score calculated by taking the mean difference of the centered log ratio (CLR) values for each family. The red color of the stacked bars represents bacterial families that were differentially abundant in each of the analysis after the FDR correction was applied.
2.5 Discussion

2.5.1 Moraxellaceae and Flavobacteriaceae Were Identified as Part of the Shared Core Microbiota in Plant- and Animal-Based Food Processing Facilities

Through comparative analyses of the microbiota found in samples collected in both plant- and animal-based food processing environments, we identified two core bacterial families: *Moraxellaceae* and *Flavobacteriaceae*. *Moraxellaceae* are comprised of three genera: *Acinetobacter*, *Psychrobacter*, and *Moraxella*. All three genera are cold tolerant, gram negative bacteria. Some members of these genera are broadly distributed in the environment, found on plants, food, soil, water, skin, and mucus membranes of animals (Teixeira & Merquior, 2014).

*Acinetobacter*, *Moraxella*, and *Psychrobacter* have all been isolated from food processing environments (Bagge-Ravn et al., 2003; Bjørkevoll et al., 2003; Frank & Hassan, 2002; Habimana et al., 2011; Stellato et al., 2015).

*Acinetobacter* has been isolated in multiple food products and food processing environments, including meat processing facilities and on beef steaks and chicken carcasses (Filippis et al., 2013; Habimana et al., 2010; Hinton et al., 2004; Russell et al., 1995; Stellato et al., 2015), fish and fish processors (Bagge-Ravn et al., 2003; Hang Wang et al., 2014; S.-P. Yang et al., 2017), milk (McHugh et al., 2021; Stellato et al., 2015), and on produce (Carvalheira et al., 2017; Lee et al., 2013). *Acinetobacter* spp. were shown to be able to form biofilms, which can have food quality and safety implications (Cherif-Antar et al., 2016; Møretrø & Langsrud, 2017; B. Wang et al., 2019). Biofilms provide nutrients for bacteria as well as interfere with sanitizer treatment due to production of extracellular polymeric substances (EPS) (Flemming et al., 2007; Wolfaardt et al., 1999). Some *Acinetobacter* species that have been isolated frequently from food...
processing environments are spoilage organisms; the most commonly studied being *A. johnsonii* and *A. lwofii* (Hinton et al., 2004; S.-P. Yang et al., 2017; Yuan et al., 2018). These two species have demonstrated lipolytic and both lipolytic and proteolytic potential at refrigerated conditions in milk (Ribeiro Júnior et al., 2018) and amine production in fish and shrimp (Kuley et al., 2017, 2019; Lakshmanan et al., 2002). The introduction of *Acinetobacter* spp. to food products is typically through environmental contamination, such as water, cross-contamination from processing equipment. *Acinetobacter* spp. can also be present on raw product itself, including the animal skin, hide, or gills (González et al., 2000; Hinton et al., 2004).

*Acinetobacter* is not typically regarded as a foodborne pathogen, as it is most commonly observed as an opportunistic pathogen in clinical settings causing an estimated 45,000 infections a year in the U.S. (Wong et al., 2017). Many of these infections are caused by *Acineteobacter* spp. that are frequently isolated from food, such as *A. lwofii* and *A. baumannii*, however infections typically occur through exposure to contaminated surfaces in the clinical environment and not through food (Wong et al., 2017). Although *Acinetobacter* spp. are not typically foodborne pathogens they have been shown to enhance adhesion and biofilm growth when cultured with *E. coli* O157:H7 (Habimana et al., 2010). Furthermore, a strain of *Acinetobacter haemolyticus* has been found to produce Shiga toxin 2 and caused bloody diarrhea in two infants in South America (Amorim & Nascimento, 2017; Grotiuz et al., 2006).

*Psychrobacter* spp. are often found in a variety of cold food processing environments and have been isolated from dairy products, meat and seafood (Barrett et al., 1986; Botta et al., 2018; Frank & Hassan, 2002; González et al., 2000; F. F. Parlapani et al., 2018; Tsironi et al., 2009). *Psychrobacter* is most commonly found in marine environments (Teixeira & Merquior, 2014). It may be introduced to the food processing environment through animals since multiple
species of *Psychrobacter* inhabit the gut of seafood animals, the respiratory tract of cattle, and the teat apex of dairy cows (Braem et al., 2012; Egerton et al., 2018; Holman et al., 2017; J. Yu et al., 2017; Yukgehnaish et al., 2020). *Psychrobacter* may also be introduced to the food processing environment through environmental contamination (Teixeira & Merquior, 2014). Species of *Psychrobacter* were reported as spoilage organisms in milk, seafood, and meat (Broekaert et al., 2013; Gennari et al., 1992; Hantsis-Zacharov & Halpern, 2007; Juni & Heym, 1986; Schmidt et al., 2012). *P. cibarius, P. imobillis, P. maritimus* have lipolytic activity and isolates of both *P. maritimus* and *P. cibarius* produce low concentrations of volatile compounds TMA, 2-butaneone, and acetone (Broekaert et al., 2013). The food spoilage that is caused by this genus has led to food loss, which inevitably results in profits lost by the food industry. *P. cibarius* isolated from a slaughter house in Denmark has been shown to form biofilms when co-cultured with different combinations of four bacteria from a food processing environment (Røder et al., 2015). Biofilm formation poses an issue when trying to remove the genus through conventional cleaning and sanitizing techniques. There have not been any reports of *Psychrobacter* causing foodborne illness.

*Moraxella* spp. are also commonly found in the food processing environment, typically in meat- (Ellis & Goodacre, 2001; Nychas et al., 2008a) and seafood processing environments (Bjørkevoll et al., 2003; Ghaly et al., 2010; Sivertsvik et al., 2002). *Moraxella* spp. enter the food processing environment on the product itself and are found in fish muscle, skin, abdominal cavity, and in cattle, goats pigs and sheep (González et al., 2000; Teixeira & Merquior, 2014). *Moraxella* spp. have food spoilage potential and are able to oxidize glucose (Eribo et al., 1985; González et al., 2000; Sperber, 2009). *Moraxella* spp. have also been shown to produce volatile compound ethyl acetate and methyl isopropyl sulfide in spoiled chicken carcasses (Freeman et
al., 1976). Strains from this genus have been reported to form biofilms in an ice cream processing environment (Gunduz & Tuncel, 2006). The ability of the Moraxellaceae family to survive at low temperature, form biofilms, and their occurrence in the environment help to explain their identification as part of the core microbiota found across plant- and animal-based food processing environments. All three genera contain bacteria that have strong biofilm forming abilities and some have even been shown to enhance biofilm growth when co-cultured with E. coli O157:H7. Although these bacteria rarely cause foodborne disease themselves, they may facilitate the survival of pathogens in the environment through biofilm formation. The development of enhanced cleaning and sanitizing routines is imperative for targeting not only the control of food pathogens, but also food spoilage organisms to mitigate the global food waste burden (Parfitt et al., 2010; Ribeiro Júnior et al., 2018).

Flavobacteriaceae is comprised of fifty-four genera (McBride, 2014). The majority of the family members are aerobic environmental bacteria (McBride, 2014). The Flavobacteriaceae family has been found in soil, water, food processing environments, as well as in fish, animals, and humans (Jooste & Hugo, 1999). The genera Weeksella, Bergeyella, and Chryseobacterium have all been isolated from dairy processing environment (Botha et al., 1998). The genus Flavobacterium is the commonly isolated member of the Flavobacteriaceae family in the food processing environment, typically found in seafood and dairy processing environments (Basson et al., 2008; Jooste & Hugo, 1999; Lafarge et al., 2004; Loch & Faisal, 2015; Sharma & Anand, 2002; Yuan et al., 2017). Flavobacterium spp. can be introduced to the seafood environment through fish tissue and water (Wiklund et al., 2000), and species F. psychrophilum, F. columnare, F. succinicans are known as fish pathogens (Cerro et al., 2002). Certain Flavobacterium spp. have a food spoilage capacity and are frequently isolated from chilled foods.
such as milk and seafood (Lafarge et al., 2004; Loch & Faisal, 2015; Waśkiewicz & Irzykowska, 2014; Yuan et al., 2018). In addition to food spoilage, Flavobacterium spp. can produce low amounts of histamine in fish, posing also a serious food safety issue (Gunaratne et al.). Flavobacterium spp., such as F. columnare and F. johnsoniae isolates, have been shown to form biofilms (Basson et al., 2008; W. Cai et al., 2013), which may facilitate their persistence in the environment. The ability of these strains to form biofilms may result in significant product loss in aquaculture as F. columnare may cause columnaris disease, which has a high mortality rate among fish (W. Cai et al., 2013, 2019; Declercq et al., 2013). Not only do strains of Flavobacterium spp. cause potential food safety issues by causing food spoilage, but they also can enhance biofilm formation by L. monocytogenes when co-cultured with this pathogen (Bremer et al., 2001). This beneficial relationship may lead to the establishment of foodborne pathogens in food processing environments colonized by Flavobacterium spp., highlighting the need for targeted cleaning and sanitizing techniques to effectively remove biofilms in food processing environments. The family’s ability to grow at low temperatures and form biofilms may be a contributing factor to their widespread nature in food processing environments.

2.5.2 Core Microbiota of Animal-based Food Processing Facilities

In addition to Moraxellaceae and Flavobacteriaceae, the families that were identified as part of the common core microbiota in animal-based food processing environments were Staphylococcaceae, Rhodobacteraceae, Lachnospiraceae, Enterobacteriaceae, and Ruminococcaceae. The rest of the identified core microbiota in animal-based facilities were unidentified families in the higher taxonomic ranks of Firmicutes, Actinomycetales, Bacteroidetes, Clostridiales, and Clostrida. The Staphylococcaceae family is made up of five
genera (Lory, 2014). Members of this family are commonly isolated from animals, human skin, dairy products, and a wide variety of environments (Gutiérrez et al., 2012; Lory, 2014; Saenz-García et al., 2020). The genus *Staphylococcus* is frequently isolated from food processing environments (Dittmann et al., 2017; Gutiérrez et al., 2012; Normanno et al., 2005; Papadopoulos et al., 2019; Xing et al., 2016). The most frequently isolated species is *S. aureus*, which is commonly isolated from food processing environments because it can be introduced through human and animal skin (Cundell, 2018; Nagase et al., 2002; Roberson et al., 1994) as well as with soil, water, and air (Savini, 2018). *S. aureus* and other species, such as *S. epidermis* are able to survive at low temperatures and form biofilms in food processing facilities (Di Ciccio et al., 2015; Miao et al., 2017; Møretrø et al., 2003; Rode et al., 2007). The high occurrence of *S. aureus* in the food industry is hazardous because some strains have the ability to produce Staphylococcal enterotoxin in food (Otto, 2014). This toxin can cause foodborne illness, resulting in an estimated 241,000 illnesses per year in the U.S. (Kadariya et al., 2014). Other *Staphylococcus* spp. also pose a threat to food safety and product loss due to spoilage in beer (Z. Yu et al., 2019) and egg processing facilities (W. Liu et al., 2021). The primary spoilage mechanism of *Staphylococcus* is lipolysis. In terms of food safety, enhanced biofilm formation was reported when strains of *S. aureus, S. capitis, and S. maltophilia* were co-cultured with *L. monocytogenes* (Di Ciccio et al., 2015; Furukawa et al., 2010; Rode et al., 2007; Z. Yu et al., 2019). Given that *S. aureus* can cause foodborne illness and potentially facilitate persistence of other foodborne pathogens in food processing facilities, the targeted control of *S. aureus* and biofilm in food processing environments could improve food safety, as well as quality.

Members of the *Rhodobacteraceae* family are aquatic bacteria (Pujalte et al., 2014) and have been found in high concentrations in crabs that failed quality inspection due to spoilage.
characteristics such as unpleasant odor (F. F. Parlapani et al., 2019), suggesting that members of this family may have food spoilage potential. However, there have not been documented cases of this family causing spoilage in the past. The cold temperatures and moisture in food processing facilities may make it a suitable environment for the microorganisms from this family to thrive (Pujalte et al., 2014). *Lachnospiraceae* and *Ruminococcaceae* have been associated with the rumen microbiome and have been commonly isolated from human and animal fecal samples (Biddle et al., 2013; Gagen et al., 2015; Ishiguro et al., 2018). This is aligned with our identification of these families as part of the core microbiota of animal-based food processing environments, as multiple studies collected samples in environments where animals were slaughtered.

*Enterobacteriaceae* are frequently isolated from humans, animals, and plants (Octavia & Lan, 2014). *Enterobacter* spp. have been isolated from infant food and milk formula, spices, sugar, eggs (Cawthorn et al., 2008; Mullane et al., 2007; Musgrove et al., 2004; Reich et al., 2010; Reichler et al., 2018) as well as meat (Castaño et al., 2002; Ojer-Usoz et al., 2013; Schill et al., 2017). Strains of *Enterobacter* spp. are able to form mature biofilms under a food processing facility conditions (Lehner et al., 2005; Huhu Wang et al., 2017). *Enterobacter sakazakii* (now taxonomically classified as *Cronobacter sakazakii*) is an opportunistic pathogens in infants (Lehner et al., 2005). This family’s ability to form biofilms and prevalence in food processing facilities, specifically dairy and meat facilities agree with the results of the identified animal-based core microbiota. The pathogenicity of *E. sakazi* (i.e., *C. sakazakii*) as well as its biofilm formation display a need for effective control of *Enterobacter* spp. in food processing environments.
The other bacterial families that were identified in the core animal microbiota have not been named to date. The families identified as part of the core bacterial families in animal-based facilities were also found to be significantly different amongst the two groups based on the ANCOM analysis. All of the families present in the animal-based core microbiota had a higher abundance in animal-based food processing environments, except for Enterobacteriaceae. Enterobacteriaceae was found to be present in a higher relative abundance in plant-based food processing environments. Based on the ALDEx2 analysis, only Ruminiconacaceae, and unclassified families from the Firmicutes and Clostridiales orders were found to be present in a higher relative abundance in animal-based environments.

2.5.3 Core Microbiota of Plant-Based Food Processing Facilities

The common core microbiota identified in plant-based food processing environments included Pseudomonadaceae, Sphingomonadaceae, Comomonadaceae, and Xanthomonadaceae, in addition to those identified as the core shared between plant- and animal-based facilities. The Pseudomonadaceae family contains the genus Pseudomonas, which is represented by well-known food spoilage microorganisms (Arslan et al., 2011; Evanowski et al., 2017; Rajmohan et al., 2002b; Raposo et al., 2016; Samuel J. Reichler et al., 2019; Tryfinopoulou et al., 2002). Pseudomonas spp. have been isolated in a multitude of food processing environments as well as in the natural environment (soil, plants, water, air) (Alonso et al., 1999; Arslan et al., 2011; Barrett et al., 1986; Caldera et al., 2016; Iglewski, 1996). Pseudomonas spp. are commonly isolated from meat (Ercolini et al., 2007; Franzetti & Scearpellini, 2007; Lavilla Lerma et al., 2015; Morales et al., 2016; Stellato et al., 2017; Huhu Wang et al., 2018), fish (Ge et al., 2017; Foteini F. Parlapani et al., 2015; Reynisson et al., 2008; Steriniša et al., 2019; Tryfinopoulou et
al., 2002), produce (Barth et al., 2009; Caldera et al., 2016; Federico et al., 2015; Pinto et al., 2015), and dairy foods (Arslan et al., 2011; Jonghe et al., 2011; Martin et al., 2011; S. J. Reichler et al., 2018; Ternström et al., 1993). The *Pseudomonadaceae* genera are typically introduced through cross contamination or on the product itself and their prevalence in food and food processing facilities is due to their psychotropic nature as well as their strong biofilm forming abilities (Allison et al., 1998; Araújo et al., 2016; Auerbach et al., 2000; Chang & Halverson, 2003; Dynes et al., 2009; Y.-J. Liu et al., 2015; Rossi et al., 2018; Stellato et al., 2017; Tolker-Nielsen et al., 2000).

*P. fluorescens, P. fragi, P. putida*, and *P. lundensis* are species frequently isolated from foods and are known to cause spoilage (Andreani et al., 2015; Barrett et al., 1986; Dosti et al., 2005; Ercolini et al., 2010; Marchand et al., 2009; Mohareb et al., 2015; Scatamburlo et al., 2015; Stellato et al., 2017; Tryfinopoulou et al., 2002). *Pseudomonas* spp. spoil dairy, meat, and produce through lipid oxidation, proteolysis, hydrolysis of triglycerides, amino acid metabolism, and/or esterification (del Olmo et al., 2018; Nychas et al., 2008; Quintieri et al., 2020; Samelis, 2006). This processes result in the production of alcohols, aldehydes, ketones, esters, volatile fatty acids, sulphur compounds, and produce pigmentation (Casaburi et al., 2015; La Storia et al., 2012; Tsigarida et al., 2003).

*Pseudomonas* spp. strains have been shown to have positive (Buchanan & Bagi, 1999; Hassan et al., 2004; Maggio et al., 2021; Saá Ibusquiza et al., 2012), neutral (Carpentier & Chassaing, 2004), and negative relationships when grown with *L. monocytogenes*, depending on the strain (Carpentier & Chassaing, 2004). Additionally, *P. fluorescens* pre-formed biofilms have been shown to be effectively colonized by planktonic *L. monocytogenes* (Puga et al., 2018). This suggests that the organism may protect the pathogen within a biofilm in food processing.
environment (Guerrieri et al., 2009; X. Pang et al., 2019; X. Y. Pang et al., 2017) and thus calls for the development of enhanced cleaning and sanitizing procedures targeted towards *Pseudomonas* spp. as well as biofilms.

*Sphingomonadaceae* are commonly isolated from freshwater, marine, roots and soil (Glaeser & Kämpfer, 2014). The genus *Sphingomonas* has been isolated from food environments and drinking water and they were shown to have biofilm forming ability and ability to grow at temperatures as low as 8°C (de Vries et al., 2019). *Sphingomonas* species are able to initiate biofilm formation (Bereschenko et al., 2010; Czieborowski et al., 2020) as well as produce EPS (Azeredo & Oliveira, 2000; Johnsen et al., 2000). They are commonly found in drinking water distribution systems due to their biofilm formation (Gulati et al., 2017; Koskinen et al., 2000). Some strains of *Sphingomonas* are able to form biofilms in these systems because of their resistance to chlorine (Sun et al., 2013). The genus has also been found to dominate conveyors in a lamb boning room (Brightwell et al., 2006). In a study that assessed the microbiome of plant- and animal-based foods, the genus *Sphingomonas* was found in all tested plant-based food samples (Jarvis et al., 2018), which agrees with its identification as part of the plant-based food processing facilities’ environment core microbiota. The species *Sphingomonas faeni*, has been shown to produce astaxanthin, which has antimicrobial activity against common food spoilage strains and strains of *L. monocytogenes* (Mageswari et al., 2015). Members of the *Sphingomonadaceae* family were detected in drinking water, roots of plants, and in plant-based foods, which aligns with our identification of this family as a core member of the plant-based food processing facility microbiota. However, the ability of some strains to inhibit *L. monocytogenes* and spoilage organisms calls for more research into potential benefits of *Sphingomonadaceae* in food processing environments.
\textit{Xanthomonadaceae} species are commonly isolated from human, marine and soil environments. \textit{Xanthomonadaceae} have been detected in multiple food processing environments (De Filippis et al., 2021; Maifreni et al., 2015; Tan et al., 2019), which agrees with its identification as a member of the plant-based facilities’ core microbiota. \textit{Xanthomonas} spp. such as \textit{X. campestris} and \textit{X. vesicatoria} have been shown to decompose produce (Banwart, 1989; Tournas, 2005) \textit{X. campestris} can also cause black rot disease in cruciferous plants (Crossman & Dow, 2004). Many strains of \textit{Xanthomonas} have been shown to form biofilms (Crossman & Dow, 2004; Sena-Vélez et al., 2015), which may also explain why the family is found in many of the plant-based facility samples. The biofilm forming abilities and spoilage potential of some species of \textit{Xanthomonadaceae} highlight a need for its further control in the environment. The \textit{Comamonadaceae} family are most commonly found in the natural environment such as soil and water as well as in plants as pathogens or in animal/ clinical samples (Willems, 2014). Species in the \textit{Comamonadaceae} have been isolated in the in dairy processing environment (Bokulich & Mills, 2013; Gil-Pulido et al., 2018), on fish and in fish production (Nedoluha & Westhoff, 1997; Pegoraro et al., 2015) and on produce (Einson et al., 2018). Members of the family are not known to cause spoilage, however \textit{Acidovorax} spp. are plant pathogens and have cause damage to food crops such as wheat roots (D. Li et al., 2011) and rice plants. (Schaad et al., 2008). The association of the \textit{Comamonadaceae} family with the natural environment and plants agrees with its identification as the member of core microbiota of plant-based food processing environments.

\textit{Pseudomonadaceae}, \textit{Sphingomonadaceae}, \textit{Comamonadaceae}, and \textit{Xanthomonadaceae} were all found to have a higher relative abundance in plant-based environments, according to the ANCOM analysis, but not the ALDEEx2 analysis. However, \textit{Sphingomonadaceae}’s mean relative
abundance in animal-based environments was 0.05% while in samples from plant-based facilities it was 0.02%.

Many of the core families identified in animal- and/or plant-based food processing environments are known to have spoilage organisms within their families. They also have strains with effective biofilm forming abilities. Pathogens such as *L. monocytogenes* have been shown to benefit when cultured with species from the families highlighted in this study, due to *L. monocytogenes*’ ability to colonize biofilms. The development of cleaning and sanitizing procedures that are focused on biofilm degradation as well as those targeting psychotrophic, Gram negative bacteria should be prioritized exemplifying the need to heighten sanitation procedures to target the core microbiota identified in this analysis.

### 2.5.4 Differential Abundance of Bacterial Families among Plant- and Animal-based Facilities

Multiple studies that have drawn conclusions about the microbiota using metagenomic sequencing in an ecosystem have analyzed the data using a multivariate approach, which may have unnecessarily removed identified taxa during normalization (G. B. Gloor et al., 2017). Both ALDEx2 and ANCOM analyses were chosen because of their complementary nature and account for the compositional nature of microbiome data (G. B. Gloor et al., 2017). ALDEx2 with an effect size (x > 1.5) cutoffs has been shown in the past to have a lower FDR than ANCOM however, it could have an excessive false negative rate (Morton et al., 2019).

Families that were identified by ANCOM as significantly different included meat spoilage organisms, gut bacteria/fecal bacteria, families that contain foodborne pathogens (e.g., *Campylobacteriaceae*), soil bacteria, root bacteria, and plant spoilage organisms/general spoilage organisms (Table 2.4.2). The differences in the sensitivity of ALDEx2 and ANCOM analyses are
demonstrated by the fact that three families were found to be differentially abundant once the effect size cutoff was factored in for the ALDEx2 analysis while 52 taxa were identified as differential abundance using ANCOM. There is currently no “gold standard” for the determination of differentially abundant taxa, however differential abundance analyses provide a general idea about what bacteria have a higher presence in the food environment (Banerjee et al., 2019; Weiss et al., 2017). The identified microorganisms should, however, be further studied to verify some of the hypotheses developed based on the comparative microbiota analyses.

As seen in the PCA (Figure 2.4.1), the samples from individual studies tended to cluster closer to each other than to samples from other studies, showing that factors other than whether or not a food processing facility produces a plant- or animal-based product affect the microbiota composition. Other likely factors that may affect the microbiota composition include air temperature, humidity, and nutrients in a facility environment, as well as location, type of facility (processing, vs. farm), and practiced sanitation procedures. It also must be noted that the areas sampled in each study were different; some studies sampled both equipment, floor, as well as discarded food samples and debris, which may have also had an impact on the identified microbiota composition and sample clustering. For example, facilities included in studies S4 and S6 both had live animals on the premise and both clustered distinctly compared to the majority of other samples and each other in the PCA plot. This suggest that having animals in the facility may greatly impact the facility environmental microbiota.

The microbiota from plant- and animal-based food processing environments were found to be different. The common core microbiota of both plant- and animal-based environments both included bacterial families with strains that are known biofilm formers and may enhance the growth of foodborne pathogens and spoilage organisms. These results suggest that the
development of cleaning and sanitizing chemicals and procedures should focus on the removal of biofilms from the food processing environment.

2.5.5 Limitations

The results were only examined at the family level to account for potential bias in taxonomic identification. However, it is unknown what specific genera or species were present in the core microbiota, limiting the conclusions we can draw from this analysis about the implications of the identified core. The bacterial families identified as part of the core microbiota were not found in every sample and the results of the core microbiota analyses may be affected by the difference in numbers of samples collected from each study. For example, study S4 collected 360 and study S6 collected 349 samples. These two studies, which both sampled animal-based environments, accounted for over 73.4% of the samples in this microbiota comparative analysis. Hence, the families identified in these papers were over-represented in the common core microbiota identified for animal-based environments. The criteria for the common core was that bacterial families needed to be present in at least 50% of all samples in said category. The common core of all studies was not explored due to the large differences in sample numbers per study. Additionally, there are no formally outlined best practices for differential abundance analyses, hence we applied two complementary methods to identify differentially abundant taxa. ANCOM has been shown in the past to be prone to false positive findings, however ALDEx2 has also been shown to have excessive false negatives compared to other methods like differential ranking, (Morton et al., 2019). Both ANCOM and ALDEx2 are considered to be more conservative than LEfSe and DESeq 2 (Lin & Peddada, 2020; Wallen, 2021). Differential abundance allows us to gain a greater understanding about what bacterial
families may have a higher presence among two environments, however, it should be further verified experimentally.
Chapter 3

Evaluating the Ability of Two Lactic Acid Bacteria Strains to Inhibit the Growth of L. monocytogenes in in vitro Tree fruit packinghouse Biofilms

3.1 Abstract

Listeria monocytogenes (Lm) is a concern in produce packing facilities because of its ability to persist through formation of biofilms with the environmental microbiome. Previous studies demonstrated lactic acid bacteria to be effective biocontrols for Lm in both lab and meat processing facilities. We aimed to evaluate the ability of lactic acid bacteria strains ATCC PTA-4761 and PTA-4759 to inhibit Lm in a monoculture, and in attached microbiota from tree fruit packinghouses.

Twenty-two phylogenetically distinct Lm isolates (10^8 and 10^7 CFU/ml) grown as microbial lawns were spot-inoculated with 1 ul of each strain and incubated for 3 days at 15, 20, 25, or 30°C. Microbiome samples from three packinghouses were inoculated into polypropylene conical tubes with Lm at 10^5 CFU/ml and either ATCC PTA-4761 or PTA-4759 at 10^7 CFU/ml, and then incubated at 15°C for 3-, 5-, or 15-days to form an attached biomass. Attached cells were removed for aerobic plate count, Lm enumeration by BAM MPN, and DNA extraction. 16s rRNA V4 amplicon sequencing of the extracted DNA was conducted using Illumina Miseq. Sequences were analyzed using Mothur v.1.44.2. Lm reductions were evaluated using ANOVA and the effect of putative biocontrol strains and growth period on quasi-biofilm composition was evaluated by PERMANOVA and differential abundance analysis.

ATCC strains PTA-4761 and PTA-4759 were identified as Enterococcus faecium and Enterococcus spp. based on whole-genome sequencing. Both strains showed significantly greater
inhibition against $10^7$ CFU/ml Lm compared to $10^8$ CFU/ml Lm lawns (P<0.0001). Lower temperatures significantly increased the ability of PTA-4759 to inhibit Lm at both concentrations (P<0.001). Lm reductions in the 15-day attached biomass were significantly greater than the positive control (P<0.01). The microbiota of the biofilms at each experiment end point were significantly different from each other. These findings may inform further studies to evaluate biological strategies for controlling *L. monocytogenes* in packinghouse environments.

### 3.2 Introduction

*Listeria monocytogenes* is one of the leading causes of foodborne illness-related deaths in the U.S. (CDC, 2018). Infection with this pathogen results in listeriosis which causes an estimated 1600 hospitalizations and 250 deaths each year (CDC, 2018). *L. monocytogenes* is commonly found in food processing facilities that are continuously cool and wet because of its ability to survive and grow in cold environments (Azizoglu et al., 2009) and its ability to form biofilms (Mazaheri et al., 2021; Piercey et al., 2016; Reis-Teixeira et al., 2017; Santos et al., 2019). *L. monocytogenes* is heat sensitive and can be inactivated in foods that undergo adequate heat treatment (Dogruyol et al., 2020; Miller et al., 2000). However, if *L. monocytogenes* is not effectively controlled in the environment, it can re-contaminate food products, especially after heat treatments, when competition from other microorganisms is low (Inmanee et al., 2019; Kozak et al., 1996; Selby et al., 2006). Contamination is particularly concerning in facilities that produce ready-to-eat food products that support growth of *L. monocytogenes* during storage.
and/or those that do not require cooking before they are consumed (Almeida et al., 2013; Iannetti et al., 2016; Kaneko et al., 1999; Kurpas et al., 2018; Pouillot et al., 2016; Stephan et al., 2015).

Fresh whole produce, including tree fruit, is commonly consumed raw and, while it generally does not support substantial growth of *L. monocytogenes*, the pathogen is capable of surviving under typical storage conditions, hence representing a food safety risk (Macarisin et al., 2019; Salazar et al., 2016; Sheng et al., 2017). There has been one outbreak and 5 recalls of tree fruit caused by *L. monocytogenes* contamination in the last five years (CDC, 2020; FDA, 2019). The listeriosis outbreak, linked to caramel coated apples, resulted in 34 hospitalizations and 7 deaths (FDA, 2015). Isolates that genometrically matched the outbreak strains were found in one of the packinghouses that supplied apples to the caramel apple processor (FDA, 2015). In the last five years, apple packing facilities have been sampled to investigate the baseline prevalence of *Listeria* spp. including *L. monocytogenes* in the pacific northwest (Ruiz-Llacsahuanga et al., 2021), southeast (Estrada et al., 2020), and northeast regions of the U.S. (Tan et al., 2019; Simonetti, et al., 2021; Chen, et al.). In the northeast, *L. monocytogenes* was found in three tree fruit packinghouse Zone 3 areas below packing lines (Tan et al., 2019). Zone numbers correspond to the level of contamination the area is exposed to; Zone 1 are food contact surfaces, Zone 2 are surfaces in close proximity to food contact surfaces, Zone 3 are non-food contact surfaces in close proximity to Zone 2 areas, Zone 4 are non-food contact areas that are not in the packing area. The occurrence of *L. monocytogenes* in facility samples ranged from 28% to 100%, depending on the facility sampled (Tan et al., 2019). In another study (Simonetti, et. al, 2021), The same packinghouses were more broadly surveyed, each at 40 standardized sites, over three successive years. In this study, 17.5% of samples tested positive for *L. monocytogenes*, most often around the packing line (19.7%), followed by cold storage rooms (16.4%), and the
packaging area (6.4%). Follow-up analyses of samples obtained from the same three facilities found a persistent strain of *L. monocytogenes* in the facility with the highest *L. monocytogenes* occurrence (Chen et al.), suggesting that adequate cleaning and sanitizing procedures were not in place. In another study that sampled eleven packinghouses in the southeast, two of which were tree fruit packinghouses, a 3.2% occurrence of *L. monocytogenes*, 2.7% occurrence of *Listeria* spp., and 0.6% occurrence of both *L. monocytogenes* and *Listeria* spp. were reported (Estrada et al., 2020). In the Pacific northwest, five commercial apple packinghouses were sampled for *Listeria* spp over two years with an overall presence of *Listeria* spp. in the 2,988 samples taken as 4.6% (Ruiz-Llacsahuanga et al., 2021). Wax coating areas, polishing brushes, dividers under fans/blowers, brushes under fans/blowers, and dry rollers had the highest occurrence of *Listeria* spp. These reports suggest that the packing line in tree fruit packinghouses is a primary source of *L. monocytogenes* contamination and that current cleaning and sanitizing practices may not be sufficient to control *Listeria* spp. and *L. monocytogenes* where there is difficult to clean equipment.

Regulatory and trade association guidelines have issued recommendations for effective control of *L. monocytogenes* and *Listeria* spp. in produce processing and packing facilities. (FDA, 2017; UFPA, 2018). Current guidance in tree fruit packinghouses include the use of physical removal of debris and soils, scrubbing of surfaces), and correct use of cleaning chemicals sanitizers. Despite these guidelines, *Listeria* spp. including *L. monocytogenes* can still be found to persist in fruit packinghouses (Estrada et al., 2020; Ruiz-Llacsahuanga et al., 2021; Simonetti et al., 2021; Tan et al., 2019). The continuing presence of difficult-to-clean equipment in inaccessible areas renders chemical cleaning and sanitizing procedures less effective as a
result of biofilm buildup over time (Colagiorgi et al., 2017; Poimenidou et al., 2016; Reis-Teixeira et al., 2017; Zameer et al., 2010).

Biofilm formation is an important strategy bacteria use to enhance survival. Biofilms are communities of microorganisms bound together on surfaces by extracellular polymeric substances (EPS) (Flemming et al., 2007). Multiple studies have shown that isolates of *L. monocytogenes* are capable of forming biofilms (Colagiorgi et al., 2017; Mazaheri et al., 2021; Piercey et al., 2016; Popowska et al., 2017; Reis-Teixeira et al., 2017; Santos et al., 2019; O. Tresse et al., 2007; O. T. Tresse et al., 2009). Biofilm formation begins with reversible attachment, as the cells continue to grow into a monolayer the cells become irreversibly attached, the cells then begin to produce EPS and grow into multilayered microcolonies (Flemming & Wingender, 2010; Renner & Weibel, 2011; Tuson & Weibel, 2013). Once the mature phase of biofilm development is reached, *L. monocytogenes* can be dispersed into the environment, hence increasing the risk for food contamination (Colagiorgi et al., 2017; Mazaheri et al., 2021; Reis-Teixeira et al., 2017). In addition to *L. monocytogenes*, other environmental microbiota found in tree fruit packinghouses, such as *Pseudomonas* (Tan et al., 2019), have been shown to form robust biofilms (Allison et al., 1998; Araújo et al., 2016; Auerbach et al., 2000; Meliani & Bensoltane, 2015; Tolker-Nielsen et al., 2000), and even to enhance the ability *L. monocytogenes* to colonize within a multi-species biofilm (Puga et al., 2018). Once a pathogen such as *L. monocytogenes* is incorporated into a biofilm, it can benefit from the protection from cleaners and sanitizers that it provides. The biofilm EPS structure serves as a physical barrier that impairs the diffusion of sanitizers (Wolfaardt et al., 1999) toward the pathogen, thus reducing its exposure to lethal levels of the sanitizer (Ibusquiza et al., 2011; Nakamura et al., 2013; Pan et al., 2006; Poimenidou et al., 2016; Rodriguez-López et al., 2018). Impaired sanitizer diffusion
therefore increases the amount exposure time needed to achieve a desired level of lethality (i.e., tolerance) (Brauner et al., 2016). *L. monocytogenes* was shown in vitro to exhibit tolerance to sanitizers, including quaternary ammonia compounds (QAC), chlorine dioxide (ClO2), benzalkonium chloride (BAC), peroxide when in multi-strain biofilms of *L. monocytogenes* (Ibusquiza et al., 2011; Pan et al., 2006). *L. monocytogenes* grown into a biofilms has also shown an increased tolerance to commercial disinfectants such as Chlorox, Quatosept, Peroxat, and Jodat (Skowron et al., 2019).

Biofilms are especially likely to form in difficult-to-clean areas in food processing environments, that allow microorganisms, including *L. monocytogenes*, to have an extended amount of contact time to attach to surfaces (Galié et al., 2018). When biofilms are present, enhanced cleaning and sanitizing procedures are necessary to re-establish control over the pathogen.

Biocontrols are microorganisms that are able to inactivate or inhibit the growth of other target microorganisms, including foodborne pathogens. Previous research has shown that strains of lactic acid bacteria (LAB) can be effective in inhibiting *L. monocytogenes* in meat processing environments (Zhao et al., 2006, 2013). A biocontrol strategy may therefore be possible to complement and enhance the standard physicochemical cleaning and sanitizing procedures in other types of processing facilities. Biocontrol mechanisms may involve production of secondary metabolites, such as bacteriocins, antibiotics, hydrogen peroxide, and organic acids, or by competitive exclusion which by generally defined as the elimination of a habitat of one of two different species with identical needs for resources. Another possible biocontrol mechanism is by infection of pathogenic bacteria by bacteriophages resulting in cell lysis and death (Alaniz Zanon et al., 2013; Bainton et al., 2004; Castellano et al., 2017; Gálvez et al., 2007; Ishaq et al., 2020;
The potential for the use of biocontrol LAB cultures to reduce pathogen levels has been widely explored for use in food preservation and safety applications (Bolocan et al., 2017; Castellano et al., 2017; Linares-Morales et al., 2018; Muriana, 1996; Pei et al., 2020; Perez et al., 2014; Yin et al., 2020). Lactic acid bacteria have been shown to significantly inhibit *L. monocytogenes* in vitro (Berrios-Rodriguez et al., 2020; Castellano et al., 2017; Rodríguez-López et al., 2018; Yin et al., 2020; Zhao et al., 2013) through competitive exclusion and production of secondary metabolites (e.g. organic acids, hydrogen peroxide, and bacteriocins) (Papagianni, 2012). Bacteriocins are proteinaceous metabolites that usually have high specificity (Gálvez et al., 2007). Successful use of biocontrols for controlling *L. monocytogenes* has been shown with the application of nisin producing LAB on food products (Hossain et al., 2020; Pérez-Ibarreche et al., 2016; Siroli et al., 2016; Zhao et al., 2004). The efficacy of LAB biocontrol strains has also been explored in commercial applications. Two lactic acid bacteria strains, *Enterococcus durans* 152 and *Lactococcus lactis* subsp. lactis C-1-152, applied to poultry processing facility drains, successfully inhibited up to 4.1 logs CFU/mL of *Listeria* spp. (Zhao et al., 2006, 2013).

The objective of this chapter is to assess whether these two biocontrol strains (*Enterococcus durans* 152 and *Lactococcus lactis* subsp. lactis C-1-152) (1) inhibit the growth of a diverse collection of *L. monocytogenes* isolates obtained from tree fruit packinghouse environments and (2) inhibit an already identified persistent strain of *L. monocytogenes* co-cultured into a quasi-biofilm using tree fruit packinghouse environmental microbiome samples.
3.3 Materials and Methods

3.3.1 Bacterial strains

Twenty-two *Listeria monocytogenes* strains were included in this study. Strains were selected to represent the phylogenetic diversity of isolates that had been collected from tree fruit packinghouses (Table 3.1). Briefly, isolates were selected based on a core genome phylogenetic tree of *L. monocytogenes* isolates collected from packinghouses to represent each phylogenetically distinct clade shown in Appendix B.2.1 and later reported in detail by Chen et al. The *L. monocytogenes* isolates were used to assess the ability of two alleged lactic acid bacteria strains, ATCC PTA-4761 (PS01156) and ATCC PTA-4759 (PS01155), to inhibit the growth of a diverse set of *L. monocytogenes* isolates obtained from tree fruit packinghouse environments. The two alleged LAB strains, identified by Zhao et al. (2013) as *Enterococcus durans* 152 (PS01156) and *Lactococcus lactis* subsp. lactis C-1-152 (PS01155) that were successfully used to control *L. monocytogenes* in a poultry processing facility (Zhao et al., 2013), were used in this study. The *L. monocytogenes* and LAB strains used in this study were stored at -80°C in brain heart infusion (BHI) broth (BD Life Sciences, Sparks, MD) supplemented with 20% glycerol. Before use, 1 µl loopful of each alleged LAB strain cryo-stock was streaked onto BHI agar (BD Life Sciences, Sparks MD) and grown for 24 hours at 35°C. Similarly, 1 µl of each *L. monocytogenes* isolate cryo-stock was streaked onto BHI agar and grown for 24 hours at 37 °C.
Table 3.2.1. *L. monocytogenes* isolates used in the study

<table>
<thead>
<tr>
<th><em>L. monocytogenes</em> Isolate</th>
<th>Packinghouse Location*</th>
<th>Isolation Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS01273</td>
<td>1</td>
<td>3/26/17</td>
</tr>
<tr>
<td>PS01274</td>
<td>3</td>
<td>1/23/17</td>
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<tr>
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<td>PS01276</td>
<td>3</td>
<td>3/7/17</td>
</tr>
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<td>PS01277</td>
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<td>3</td>
<td>1/13/17</td>
</tr>
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<td>11/17/16</td>
</tr>
<tr>
<td>PS01280</td>
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<td>1/23/17</td>
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<td>2/6/17</td>
</tr>
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</tr>
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</tr>
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<td>2/6/17</td>
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</tr>
<tr>
<td>PS01295</td>
<td>2</td>
<td>10/19/16</td>
</tr>
</tbody>
</table>

*Packinghouse number, packinghouses were all located in the Northeast

### 3.3.2 Verification of Taxonomic Identity of Two Alleged Lactic Acid Bacteria Strains

The two alleged LAB strains PS01156 and PS01155, previously identified as *E. durans* and *L. lactis* species respectively, were chosen for this study because of their reported ability to inhibit *L. monocytogenes* (Zhao et al., 2004, 2006, 2013). To verify the authenticity biological material obtained from ATCC we carried out, 16S rRNA PCR amplification using primers PEU7_F (5’- GCA AAC AGG ATT AGA TAC CC -3’) (Rothman et al., 2002), and DG74_R (5’- AGG AGG TGA TCC AAC CGC A –3’) (Fromm & Boor, 2004). The PCR amplicons were
purified using ExoSAP treatment and sent for Sanger sequencing to the Penn State Genomics Core Facility. Sanger sequences were analyzed using Ape v. 5.4-1 (Paradis & Schliep, 2019) and phylogenetically compared with type strains of *Enterococcus* and *Lactococcus* species. The full length 16S rRNA sequences of type strains were downloaded from the Ribosomal Database Project (RDP) (Cole et al., 2014). Sequences of type strains were phylogenetically compared with those of strains PS01156 and PS01155 by constructing a maximum likelihood tree, using a GTR Gamma substitution model, and 1000 bootstrap repetitions in MEGA X v. 0.1 (Kumar et al., 2018). The phylogenetically closest type strains did not in fact belong to the species reported in Zhao et al. (Zhao et al., 2013). Therefore, the two strains of interest were re-obtained from ATCC to evaluate whether the discrepancy in genus identification was due to laboratory contamination. The 16S rRNA analyses were repeated and the same results were obtained with the new stocks of LAB strains. Hence, we proceeded with whole-genome sequence (WGS)-based strain identification.

The two new LAB strains were grown in BHI broth again as already described. DNA was then extracted using the E.Z.N.A Bacterial DNA Kit (Omega bio-tek, GA) following the manufacturer’s procedure. The extracted DNA was quantified using Nanodrop One (ThermoFisher, Wilmington, DE) and Qubit 3 (ThermoFisher, Foster City, CA), and stored at -80°C until it was sent to Novogene Bioinformatics Institute (Beijing, China) for whole-genome sequencing. Samples first underwent quality control using agarose gel electrophoresis to verify the DNA integrity, followed by Qubit 2.0 (ThermoFisher, Foster City, CA) to determine the DNA concentration. Library construction was then performed first by randomly fragmenting the genomic DNA using sonication. Fragments were then end polished, A-tailed, and ligated with full length adapters for Illumina sequencing. Prepared libraries were purified with AMPure XP
beads. The library fragment size distributions were verified using Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). Libraries were quantified by real-time PCR to ensure that the criterion of 3nM was met, and then pooled. Illumina Novaseq 6000 (Illumina, San Diego, CA) was used for 150 bp paired-end sequencing. The read quality was then assessed using Fastqc v. 0.11.1 (Wingett & Andrews, 2018) and Trimmomatic v. 3.36 (Bolger et al., 2014). Reads were then assembled de novo using SPAdes v. 3.13.1 (Bankevich et al., 2012), with k-mer lengths of 99 and 127 bp (Cha & Bird, 2016) and the careful option to reduce mismatches and short indels. The quality of the assembled reads was assessed using Quast v. 4.6.1. (Gurevich et al., 2013) by calculating assembly quality metrics such as N50, GC content and total number of contigs. Burrows Wheeler Alignment tool (BWA) v. 0.7.12 (H. Li & Durbin, 2009), and Samtools v. 1.5 (H. Li et al., 2009) were used in order to calculate average draft genome coverage. The assembled genomes were then submitted to the Type Strain Genome Server to identify taxonomic species (Meier-Kolthoff J.P., Göker M., 2019). If the identity was deemed to be unreliable by the Type Strain Genome Server, the strains’ genomes were also analyzed using the Genome-to-Genome Distance Calculator (Camacho et al., 2009; Lagesen et al., 2007; Meier-Kolthoff et al., 2013; Ondov et al., 2016). Draft genomes were then analyzed using BAGEL4 on the web server (Heel, A., 2018) to identify potential bacteriocin-encoding genes. Finally, the sequencing reads and the assembled genomes were submitted to the NCBI SRA and the genome database through the NCBI Prokaryotic Annotation Pipeline, respectively (Tatusova et al., 2016).
3.3.3 Microbiome Sampling and Processing

Environmental microbiome samples were collected over two visits to three packinghouses. Samples were taken underneath the roller brush conveyor in the wash, dry, and wax sections of the packing lines. Samples were collected using three hydrated sponges with a D/E neutralizing broth (3M, St. Paul, Minnesota) from a 40 x 40 cm surface on April 4th and 24th of 2019 (Tan et al. 2019). Each sample was supplemented with 90 mL of BHI broth and stomached for 7 minutes at 260 rpms (Seward, United Kingdom). Samples collected from the same facility were combined in 500 mL sterile glass bottles to create composite samples representative of each facility. 100 mL of sterile glycerol was added to each of the composite samples. Samples were thoroughly mixed and five 50-mL aliquots were added to sterile conical tubes (VWR, Radnor, PA), and stored at -80 °C until further use. The frozen microbiome samples were thawed at room temperature for one and a half hours prior to use in experiments.

3.3.4 Spot Inoculation Assay for Evaluation of Anti-Listerial Activity of Two Alleged LAB Strains

In order to evaluate the anti-Listerial activity of PS01156 and PS01155, bacterial lawns were developed from the twenty-two phylogenetically diverse L. monocytogenes isolates (Table 3.2.1). PS01156 and PS01155 and the twenty-one L. monocytogenes strains were grown on BHI agar at 35 or 37°C for 24 +/- 2 hours, respectively. Colonies of the L. monocytogenes isolates were then suspended in 1 x PBS (0.8% NaCl, 0.02% KCl, 0.144% Na2HPO4, 0.024% KH2PO4, pH adjusted to 7.4 using 1M HCl) and diluted to a concentration of 1 x 108 and 1 x 107 CFU/ml. For each L.
monocytogenes isolate, the inocula at the two respective concentrations were swabbed onto two separate BHI agar plates using a sterilized cotton tip swab (Puritan, Guilford, ME) in a continuous movement to prepare the lawns.

The LAB strains PS01156 and PS01155 colonies were also suspended in PBS to prepare an inoculum containing 1 x 10^8 CFU/ml. The lawns were spot inoculated with one µl each of PS01156 and PS01155 in duplicate for incubation at 15, 20, 25, and 30°C for 96 hours (Holt, John G., 1860). Due to the lack of uniformity of the inoculated biocontrol spot (e.g., undulate edge of the inoculum), the zone of inhibition was measured from the outer edge of the inoculated culture to the outer edge of the zone of inhibition at three different locations. The three measurements were averaged, and the average was reported as a zone of inhibition.

3.3.5 Bacteriocin Partial Isolation

To further understand the mechanism by which the two LAB strains inhibit L. monocytogenes, and to evaluate the potential contribution of bacteriocins to the inhibition, we evaluated the anti-Listerial activity of cell-free supernatants from each biocontrol strain. To assess the effect of different growth conditions on the production of inhibitory compounds strains PS01156 and PS01155 were grown at 37°C for 24 and 48 hours in De Man Rogosa and Sharpe (MRS) broth at a pH of 6.2 and in BHI broth at a pH of 6.2 and 7, following the growth conditions reported by in Yang et al., 2018 (Yang et al., 2018). Additionally, the strain PS01156 was grown in MRS broth at 41.5°C for 12 hours, 25°C for 24 hours (Luis M. Cintas et al., 2000), and at 37°C in TSB broth at a pH of 7.2 and 6.2 (Du et al., 2017), because the supernatant of the culture grown in BHI and MRS did not inhibit L. monocytogenes lawns. Bacteriocin isolation
followed the methods used in Yang et al. (E. Yang et al., 2018). Briefly, at each time point, the cultures were centrifuged at 8,000 g for 20 minutes and then filtered using a 0.2 µm cellulose filter (VWR, China Cat No. 28145-477). The filtered supernatant was neutralized using 1 M NaOH to evaluate the contribution of organic acids to the inhibition of *L. monocytogenes*. The neutralized supernatant was further treated with 1 mg/ml catalase from bovine liver (Sigma Aldrich, St. Louis, MO) for 30 minutes at 25°C, to evaluate relative contribution of hydrogen peroxide to the inhibition of *L. monocytogenes*. Finally, a catalase-treated supernatant was treated with proteinase K for 2 hours at 37 °C to determine whether the residual antilisterial compounds in the catalase-treated supernatant are proteinaceous. A disk diffusion assay was performed in order to assess the ability of supernatants to inhibit *L. monocytogenes* isolate PS01273. *L. monocytogenes* lawns were prepared as described above. After each treatment, 25 µl of supernatant was applied to a sterile disk (Hardy Diagnostics, Santa Maria, CA) and applied to a 1 x 10^7 CFU/mL lawn of *L. monocytogenes*. Lawns were then incubated at 30°C for 24 and 48 +/- 2 hours. Inhibition zones were then measured from the outer edge of the disk to the outer edge of the zone of inhibition. The incubation temperature, pH, and broth medium in which strains PS01156 and PS01155 produced the largest quantities of inhibitory compounds after incubation for 24 hours used to grow the PS01156 and PS01155 strains for the quasi-biofilm assay.

### 3.3.6 Quasi-biofilm Assay

The effects of PS01156 and PS01155 on the survival and growth of *L. monocytogenes* in tree fruit packinghouse environmental microbiome samples were evaluated by co-culturing them into a quasi-biofilm. We here define a quasi-biofilm as an
attached biomass for which the production of extracellular polymeric substances (EPS) and a three-dimensional structure characteristic of a biofilm has not been verified. Fifteen milliliter polypropylene conical tubes (VWR Cat. No. 89039-664) were used to grow quasi-biofilms. This method provided single use, clean, unscathed surfaces to limit the impact of external, uncontrolled factors on the formation of the quasi-biofilm. For both PS01155 and PS01156, a single colony was grown for 24 hours on BHI agar, suspended in MRS broth (pH 6.2), and grown for 24 hours at 37°C (E. Yang et al., 2018). *L. monocytogenes* isolate PS01273 was used in the quasi-biofilm assay and

For each of the 3-, 5-, and 15-day quasi-biofilm experiments there were three sets of facility samples. Each set included a negative control, positive control, PS01155 treatment, and PS01156 treatment. Three technical replicates were performed for each facility environmental microbiome sample set. The final volume in each tube of the assay was 2 ml. For each set of assays, the negative control tubes had 2 mL of facility samples. The positive control tube had 1.8 mL of facility samples and 0.2 mL $1 \times 10^6$ *L. monocytogenes* PS01273. The PS01155 treatment tubes had 1.6 mL of facility samples, 0.2 mL of $1 \times 10^6$ PS01273, and 0.2 mL of $1 \times 10^8$ PS01155. The PS01156 treatment tubes had 1.6 mL of facility samples, 0.2 mL of $1 \times 10^6$ PS01273, and 0.2 mL of $1 \times 10^8$ PS01156. Additionally, a fifth tube, with just 2 ml BHI broth, was added to each set of assays to serve as a sterility control to detect potential cross-contamination. This resulted in each independent experimental replicate included a set of three negative control tubes (just environmental microbiome sample), three positive control tubes (environmental microbiome sample and $1 \times 10^5$ CFU/mL *L. monocytogenes*), three PS01155 and three PS01156 treatment tubes (environmental microbiome sample, $1 \times 10^5$ CFU/mL *L.*
monocytogenes, and 1 x 10^7 CFU/mL of the strain PS01155 or PS01156). This resulted in a relative difference in the starting biomass of about 0.2 logs, in the negative controls when compared to the positive control and two LAB tubes. Total microorganisms were not standardized among the NC, PC, and two LAB treatment tubes.

On the assay start day (day 0), environmental microbiome samples were serially ten-fold diluted in PBS for aerobic plate count (APC) and L. monocytogenes enumeration. BHIA was used for aerobic plate count and ALOA was used for L. monocytogenes enumeration using BAM MPN method. Each series of assays was incubated for 3-, 5-, or 15- days at 15°C to grow the quasi-biofilms. This incubation temperature was selected to mimic the temperature in tree fruit packinghouses.

Strains PS01155 or PS01156 were added to the environmental microbiome just on day 0 in a 3-day and 5-day experiment. In a 15-day experiment, the attached cells underwent rinsing and reapplication of strain PS01155 or PS01156, developed based on Zhao et al. 2013, to evaluate whether reapplication resulted in additional reduction of L. monocytogenes (Zhao et al., 2013). Briefly, on days 5 and 10 of the 15-day experiment, detached cells were removed, and the attached biomass was washed twice with 2 ml of sterile 1 x PBS. Strains PS01155 or PS01156 were then reapplied at the same concentration as on day 0. This procedure differed from the Zhao el al. 2013 study, in that the biofilms were cleaned, and LAB biocontrol strains were reapplied every 5 days. In the paper Zhao et. al, 2013, their reapplication of the LAB treatment was every day for a week followed by twice a week for the following 3 weeks (Zhao et al., 2013). Our research team decided on the 5-day intervals due to the insignificant change in L. monocytogenes concentration observed in the 5-day quasi-biofilms.
3.3.7 Quantification of Aerobic Mesophilic Microorganisms and *L. monocytogenes*

On the last day of incubation for all assays (day 3, day 5, and day 15), the detached cells were removed from the tubes and the attached biomass was washed twice with 2 ml of sterile 1x PBS. The attached biomass was then detached by adding to each sample an additional 2 mL of PBS, 1 g of sterile glass beads with a 2 mm diameter (MP Biomedicals, Hessen, Germany) and vortexing for 2 minutes and 30 seconds. One milliliter of each detached biofilm sample was used for DNA extraction and the other milliliter of the samples was serially, ten-fold diluted in 1 x PBS for total aerobic plate count and *L. monocytogenes* quantification. Dilutions were plated on BHI agar for aerobic plate counts in order to determine total aerobic organisms. The dilutions were also used for the FDA BAM-based most probable number (MPN) assay in order to determine the concentration of *L. monocytogenes*. Sample dilutions were spread-plated in triplicate on BHI agar and incubated at 37 °C for 24 and 48 +/- 2 hours. BHI agar and an incubation temperature of 37 °C were chosen over plate count agar and a standard incubation temperature of 35 °C (FDA, 2020) because it resulted in better growth of total microorganisms, as determined in preliminary experiments (data not shown). Sample dilutions were spread-plated in triplicate on BHI agar and incubated at 37 °C for 24 and 48 +/- 2 hours. The plates that had between 30 - 300 CFU per plate were counted, and the CFU/tube were calculated by averaging replicate counts.

*L. monocytogenes* was enriched and quantified according to the Food and Drug administration Bacteriological Analytical Manual (BAM) Detection and Enumeration of *Listeria monocytogenes* (FDA, 2019b) and BAM Most Probable Number from Serial Dilutions (FDA,
Briefly, 100 µl of each dilution were inoculated into three microcentrifuge tubes pre-filled with 900 µl of buffered *Listeria* enrichment broth (BLEB) (Hardy Diagnostics, Santa Maria, CA). Inoculated microcentrifuge tubes were incubated at 30°C for 4 hours. After 4 hours of incubation, 4 µl of BLEB supplement (10 mg/L acriflavin, 50 mg/L nalidixic acid, and 40 mg/L cycloheximide; Sigma Aldrich, St Louis, MO), was added to each sample and the tubes were further incubated at 30 °C for additional 44 +/- 2 hours. After incubation, one loopful of each MPN dilution (from 1 x 10^-2 through 10^-9) was streaked in triplicates onto Agar *Listeria* Ottavani & Agosti (ALOA) agar plates (BioRad, Marnes la Coquette, France). Inoculated plates were incubated for 24 and 48 hours at 37 °C. After 24 and 48-hour incubation, plates were examined for growth of blue-green colonies with a halo. Each dilution replicate that had blue-green colonies with a halo was considered positive for *L. monocytogenes* growth. The BAM MPN calculator downloaded from the FDA BAM website (FDA, 2019) was used to calculate the MPN/tube. The significance of *L. monocytogenes* reduction for each assay was assessed by performing a one-way ANOVA and Tukey’s HSD test using a package stats v. 4.0.3 in R v. 3.6.1 (R Core Team, 2020). Standard error was calculated for technical replicates from each independent experiment based on the standard deviation in R v. 3.6.1.

### 3.3.8 DNA Extraction for Microbiota Sequencing

One ml of each detached quasi-biofilm sample was centrifuged at 13,000 g for 20 minutes (Eppendorf, Hamburg, Germany). The resulting supernatant was discarded, and the pellets were stored at -80°C until DNA extraction using DNeasy Power Biofilm Kit (Qiagen, Germantown, MD) following the manufacturer’s protocol. The extracted DNA was quantified
spectrophotometrically using Nanodrop One (ThermoFisher, Wilmington, DE) and fluorometrically using Qubit 3 (ThermoFisher, Foster City, CA), with a dsDNA high sensitivity assay kit. Extracted DNA samples were stored at -80°C until they were sent for 16S rRNA amplicon sequencing to determine the composition of microbiota at the start and end of each quasi-biofilm experiment.

3.3.9 16s rRNA V4 Amplification, Amplicon Library Preparation, and Amplicon Sequencing

16s rRNA V4 amplification, library preparation and Illumina Novaseq 6000 (Illumina, San Diego, CA) sequencing were carried out by Novogene Bioinformatics Institute (Beijing, China). Briefly, 16S rRNA V4 PCR amplification was performed by PCR using a forward primer 515F (5’GTGCCAGCMGCCGCGTA3’) and a reverse primer 806R (5’GGACTACHVGGGTWTCTAAT3’) (Apprill et al., 2015; Caporaso et al., 2012; Parada et al., 2016) with barcodes. DNA libraries were constructed by end repairing, adding A’s to tails, followed by purification. The DNA libraries were sequenced using an Illumina Novaseq 6000 to generate 250 bp paired-end reads.

3.3.10 Sequence Analyses

Sequences were analyzed using Mothur (v. 1.44.2) (Schloss et al., 2009) by following the MiSeq standard operating procedure (Kozich et al., 2013). First, contigs were created by generating a reverse complement of the reverse reads. The reads were then joined into contigs by aligning the pairs of sequences and identifying positions of disagreement. Contigs that were less
than 251 bp long, more than 253 bp long, or had ambiguous bases were excluded from further processing. The remaining sequences were then aligned to the SILVA database v. 132 and sequences that aligned before or after the target site of alignment were discarded. The sequences were then de-noised and chimera sequences were removed. Taxonomy was assigned using the SILVA database v.132. Sequences that were identified as archaea, eukaryotes, chloroplasts, mitochondria, or remained unclassified, were discarded. The remaining bacterial sequences that were 97% or more similar were assigned the same OTU using the OptiClust method. Finally, the taxonomy was assigned to OTUs.

### 3.3.11 OTU Normalization and Principal Component Analysis

The OTU table was normalized in R v. 3.6.1 using a Bayesian multiplicative replacement of count zeros, specifically the count zero multiplicative method. The count zero method uses a nonparametric simple multiplicative replacement on the matrix of estimated probabilities to replace any sampling zeros in the OTU table (Martín-Fernández et al., 2015; Xia et al., 2018). The normalized counts were converted to relative abundances and subjected to centered log transformation (clr) using R package zCompositions v. 1.3.4. (Xia et al., 2018). Singular value decomposition was then used to perform principal component analysis in order to evaluate whether samples cluster by facility, quasi-biofilm growth period (3-day, 5-day, or 15-day experiment), or treatment (with and without the addition of strains PS01155 or PS01156). R packages vegan v. 2.5.7, compositions v. 2.0.0, and ggplot2 v. 3.3.3 were used to plot the first two principal components in order to visualize sample clustering. Additionally, relative abundance bubble plots were generated to visualize the most abundant taxa in each growth period experiment and facility using ggplot2 v. 3.3.3.
3.3.12 PERMANOVA and Differential Abundance Calculation

PERMANOVA analysis was performed based on the calculated Aitchinson distances to test whether there are significant differences in the microbiota composition among end of experiment samples from (i) different quasi-biofilm growth period experiments, (ii) different facilities, and (ii) different treatments (NC, PC, PS01155 or PS01156). PERMANOVA analyses were carried out using an R package pairwiseAdonis v. 0.0.1

Differential abundance (DA) analysis was used to identify OTUs that were differentially abundant in samples grown in 3- vs. 5-day quasi-biofilms, 5- day vs. 15-day quasi-biofilms and 3-day vs 15-day quasi-biofilms. ALDEx2 was used to calculate differential abundance (Fernandes et al., 2014; G. B. Gloor et al., 2016) at the OTU level. This method was chosen because it is appropriate for compositional data analysis and has been shown to minimize the false discovery rate (FDR) (Fernandes et al., 2014; G. Gloor, 2018). In the process of ALDEx2 differential abundance analysis, 128 Monte Carlo instances (random samples of the relative abundance of each OTU) were sampled from a Dirichlet distribution for each quasi-biofilm sample. Each instance was then transformed using clr (Xia et al., 2018). Differentially abundant taxa were identified using Welch’s t and Wilcoxon rank sums test, with a Benjamini-Hochberg correction, followed by the application of an effect size cutoff of +/- 1 (G. Gloor, 2018) using an R package ALDEx2 v. 1.22.0, by adapting the script published by Xia et al. (Xia et al., 2018). The methods used in this analysis can be found at https://github.com/pcs5238/quaisbio.
3.4 Results

3.4.1 Strains PS01155 and PS01156 were Identified as Enterococcus faecium and Enterococcus lactis.

The two alleged lactic acid bacteria (LAB) strains, PS01155 and PS01156, were purchased from ATCC as Lactococcus lactis subsp. lactis C-1-152 and Enterococcus durans 152, respectively. The strains were selected because they were reported to have significantly reduced L. monocytogenes over the course of 34 weeks in the drains of a poultry processing facility (Zhao et al., 2013). The strain identity was not guaranteed by ATCC, hence we first carried out taxonomic identification of strains PS01155 and PS01156. The maximum likelihood tree built based on the 16S rDNA sequences of PS01155 and PS01156 and type strains of Enterococcus and Lactococcus genera indicated that the strains PS01155 and PS01156 were closely related to each other and to Enterococcus facieum and Enterococcus lactis (Appendix Fig B.3.1). To exclude the possibility of laboratory contamination, the two strains, PS01155 and PS01156, were re-ordered from the ATCC. Sanger sequencing was repeated (Appendix Fig B.3.1), and the same results were obtained.

To confidently identify strains PS01155 and PS01156, whole genome sequencing was carried out. Sequencing reads of PS01155 and PS01156 genome's were assembled in 103 and 159 contigs, respectively. The average coverage was 400.183 and 441.864 and the total length of the genomes was 2767103 and 2852918 bp, respectively. Sequencing reads and assemblies were deposited in the NCBI under the BioProject PRJNA670330, and sequence accession numbers SAMN16493025, SAMN16493026.
The taxonomic identity of PS01155 and PS01156 was determined using the Type Strain Genome Server (Meier-Kolthoff et al., 2013). The strain PS01155 was identified as *Enterococcus faecium*, not *Lactococcus lactis*. The strain PS01156 was identified as *Enterococcus lactis*, not *Enterococcus durans*. The confidence in taxonomic identification was evaluated based on the d scores, where \(d_0\) score indicated the length of all high scoring segment pairs (HSPs) divided by total genome length. The \(d_4\) score indicated the sum of all identities found in HSPs divided by overall HSP length. The \(d_6\) score indicated the sum of all identities found in HSPs divided by total genome length (Meier-Kolthoff et al., 2013). The identification of PS01155 revealed a \(d_0\) score of 90.8, a \(d_4\) score of 94.2 and a \(d_6\) score of 94.2, PS01156 and a \(d_0\) score of 28.7, a \(d_4\) score of 88.1, and a \(d_6\) score of 31.7. Based on the recommendations of Meier-Kolthoff et al. (2013), the PS01156 identification required verification due to the substantial difference in \(d_0\), \(d_4\), and \(d_6\) values. The PS01156 was therefore submitted to the Genome-to-Genome Distance Calculator (Meier-Kolthoff et al., 2013). This tool was used to compare the genome of the strain PS01156 with publicly available complete whole-genome sequences of strains of *Enterococcus lactis*, deposited under accession numbers: JAFDUG000000000, JAENKN000000000, JADZMB000000000, JACEIT000000000, WOTS00000000, WOTT00000000, WOTR00000000, WOTQ00000000, WOTP00000000. The PS01156 was found to be most closely related to reference genome JAENKN000000000, having \(d_0\), \(d_4\), and \(d_6\) values of 88.8, 94.5, and 92.3. This result suggested that the PS01156 is *Enterococcus lactis*.

The draft genomes of strains PS01155 and PS01156 were also submitted to BAGEL4 web server to detect bacteriocin genes (van Heel et al., 2018). In the strain PS01155, BAGEL4
detected genes for enterocinB, enterocin SE-K4, enterolysin A and enterocin A. In the strain PS01156, BAGEL4 detected genes for enterocin P, enterocin L50b, enterolysin, A and UviB.

3.4.2 Ability of strains PS01155 and PS01156 Bacteria Strains to inhibit L. monocytogenes

The strains PS01155 and PS01156 were able to inhibit all 22 L. monocytogenes isolates they were tested against in the spot inoculation assay. The ability of the LAB strains to inhibit L. monocytogenes lawns at 15°C was only assessed for 21 strains, excluding, PS01281. The average zone of inhibition for each strain, grown at four different temperatures, and tested against two different concentrations of each L. monocytogenes isolate are shown in Figure 3.4.1.
Figure 3.4.1 Inhibition of *L. monocytogenes* by PS01155 (A) and PS01155 (B), N=22 for temperatures 20, 25, and 30°C and N=21 for 15°C, n=2. The green bars represent the average zone of inhibition (mm) observed on the $10^8$ CFU/mL *L. monocytogenes* lawns at 15, 20, 25, and 30 °C, with standard error bars. The blue bars represent the average zone of inhibition observed on the $10^7$ CFU/mL *L. monocytogenes* lawns at 15, 20, 25, and 30 °C, with standard error bars. Both strains produced significantly larger inhibition zones on the $10^7$ CFU/mL *L. monocytogenes* lawns (PS01155, p = 1.13^{-8}; PS01156, p = <2.2^{-16}). The ability of PS01156 to
inhibit *L. monocytogenes* lawns was impacted by the temperature at both concentrations (10⁷ CFU/mL, p = 0.0000102; 10⁸ CFU/mL, p = 0.00125). The strain PS01156 produced larger inhibition of *L. monocytogenes* at 15°C when compared to the inhibition at 20, 25, and 30°C (Appendix Table B.3.2). The inhibition of 10⁸ CFU/mL *L. monocytogenes* lawns by PS01156 was greater at 15°C, 20°C, and 25°C compared to the inhibition observed at 30°C (Appendix Table B.3.2). Temperature did not have a significant effect on the ability of the strain PS01155 to inhibit *L. monocytogenes* lawns at either concentration. However, numerically, the inhibition of 10⁷ CFU/mL lawns by PS01155 was greatest at 15 °C. Both stains exhibited similar abilities to inhibit the different *L. monocytogenes* strains at both concentrations (10⁷ CFU/mL, p = 0.664; 10⁸ CFU/mL, p = 0.745).

### 3.4.3 Bacteriocin Partial Purification

The strains PS01155 and PS01156 were initially grown at 35°C in BHI broth, however the filtered supernatant from both strains were unable to inhibit *L. monocytogenes* when tested on 10⁷ CFU/mL *L. monocytogenes* lawns of PS01273 (n=2). The other growth temperatures and media used to grow the strains for partial bacteriocins isolation in Yang et al. (2018) were then used to assess the ability of PS01155 and PS01156 to produce bacteriocin-like, proteinaceous secondary metabolites. The conditions selected from Du et al. (2017) and Cintas, et al. (2000) were also used to assess bacteriocin production by strain PS01156.

Supernatant from PS01155 inhibited the *L. monocytogenes* lawn at each step until the Proteinase K treatment, confirming that the inhibition of *L. monocytogenes* by the neutralized, hydrogen peroxide-free supernatant was due to proteinaceous compounds (Figure 3.4.2). For each growth condition, the zone of inhibition decreased after the removal of organic acids and
hydrogen peroxides. This suggests that inhibition due to proteinaceous bacteriocins is not the only mechanism by which PS01155 can inhibit *L. monocytogenes* (Figure 3.4.2). The supernatant of PS01156 inhibited *L. monocytogenes* lawns only when the strain was grown in MRS at 37°C for 48 hours, but inhibition was lost after treatment with catalase (Figure 3.4.2), suggesting that the inhibition observed was not due to bacteriocins. PS01156 was then grown in MRS broth at 41.5°C for 12 hours, 25°C for 24 hours (Luis M. Cintas et al., 2000), and at 37°C in TSB broth at a pH of 7.2 and 6.2 (Du et al., 2017). These conditions were selected based on the bacteriocins enterocin P and enterocin L50b that were identified in the BAGEL4 analysis. PS01156 was grown twice at these conditions but did not inhibit the *L. monocytogenes* lawn.

Figure 3.4.2. Average zone of inhibition by LAB isolate supernatants at each partial isolation step when grown at 37°C in De Man Rogosa and Sharpe (MRS) broth. The partial isolation of bacteriocins assay was performed twice at each temperature. The yellow bars represent the zone of inhibition (cm) for filtered supernatant, the green bars represent the zone of inhibition for neutralized supernatant, and the purple bars represent the zone of inhibition for catalase-treated supernatant.
3.4.4 Ability of PS01155 and PS01156 to Inhibit L. monocytogenes in a Quasi-biofilm

In both the 3- and 5-day quasi-biofilm assays, *L. monocytogenes* was not significantly reduced in the samples to which PS01155 or PS01156 strain were added. However, in the 3-day quasi-biofilm assays, *L. monocytogenes* was numerically reduced by over 1 log CFU/mL when strains PS01155 or PS01156 were added to the environmental microbiomes from facility 1 (F1) and facility 3 (F3). In microbiome samples from F1 and F3, the addition of the strain PS01155 reduced *L. monocytogenes* by 1.30 and 1.48 log CFU/mL, respectively (Figure 3.4.3A). The addition of PS01156 reduced *L. monocytogenes* by 2.19 and 2.14 log CFU/mL, respectively (Figure 3.4.3C). In microbiome samples from F2, the addition of PS01155 and PS01156 reduced *L. monocytogenes* by 0.291 and 0.211 log CFU/mL, respectively (Figure 3.4.3B).
Figure 3.4.3: Aerobic plate count and *L. monocytogenes* concentration in the 3-day quasi-biofilms grown from environmental microbiomes collected from facilities F1 (A), F2 (B), and F3 (C). The number overlayed on each bar is the average *L. monocytogenes* concentration in each type of quasi-biofilm. The orange bars represent a standard error of the *L. monocytogenes* concentration. The line above the bars represents the average aerobic plate count in each sample and the bars indicate the standard error. The average value is shown above the error bars. The dotted line represents the limit of detection of the BAM MPN method, which is 1.522 log MPN/mL.

Aerobic plate counts were consistently greater than the concentration of *L. monocytogenes* in each tested sample. For the 3-day quasi-biofilms aerobic plate counts were highest in samples with PS01155 or PS01156 added, regardless of which facility the microbiome sample originated from and despite the reduction in *L. monocytogenes* concentration in samples from F1 and F3. In the 5-day quasi-biofilms, *L. monocytogenes* reductions were less than 1 log CFU/mL in all samples treated with either strain (Figure 3.4.4 A, B, C).
Figure 3.4.4: Aerobic plate count and *L. monocytogenes* concentration in the 5-day quasi-biofilms grown from environmental microbiomes collected from facilities F1 (A), F2 (B), and F3 (C), respectively. The number overlayed on each bar is the average *L. monocytogenes* concentration and the bars indicate the standard error. The line above the bars represents the average aerobic plate count at the bars indicate standard error. The average value is shown above the error bars. The dotted line represents the limit of detection of the BAM MPN method, which is 1.522 log MPN/mL.

In the 5-day quasi-biofilms, microbiome samples from F2 that were supplemented with the strain PS01155, the concentration of *L. monocytogenes* increased within the quasi-biofilm,
however, this increase was not statistically significant (Figure 3.4.4B). For five of the 5-day quasi-biofilm aerobic plate counts (F2PC, F1Ef, F1El, F2Ef, F3NC) counts were greater than 300 CFU/mL, resulting in uncountable plates.

The 15-day quasi-biofilms were performed after the completion of the 3- and 5-day quasi-biofilm assays. Due to the insignificant change in *L. monocytogenes* concentrations after 5-days of growth with PS01155 and PS01156, the additive effect of the strains was assessed in a 15-day quasi-biofilm assay with repeated PS01155 and PS01156 culture addition. *L. monocytogenes* was significantly reduced by the strains PS01155 and PS01156, as measured on in a quasi-biofilm on day 15 (PS01155, p = 0.004; PS01156, p = 0.002) (Figure 3.4.5 A, B, C). However, the samples treated with PS01155 and PS01156 had *L. monocytogenes* reductions less than 0.50 log CFU/mL. This is not an ideal log kill for the control of *L. monocytogenes* in tree fruit packinghouses because concentrations as high as 5 log CFU/mL have been observed in the environment (Tan et al., 2019).
Figure 3.4.5: Aerobic plate count and *L. monocytogenes* concentration in the 15-day quasi-biofilms grown from environmental microbiomes collected from facilities F1 (A), F2 (B), and F3 (C), respectively. The number overlayed on each bar is the average *L. monocytogenes* concentration and the bars indicate the standard error. The line above the bars represents the average aerobic plate count ant the bars indicate the error. The average value is shown above the error bar. The dotted line represents the limit of detection of the BAM MPN method, which is 1.522 log MPN/mL.
Aerobic plate counts were 1-3 log CFU/mL greater than the *L. monocytogenes* concentrations for each sample. For three of the samples (F2 with added PS01155, F1 positive control, and F2 with added PS01156), the aerobic plate count average was calculated based on just two plate count replicates due to third replicates plates being uncountable (having larger than 300 CFU/plate).

### 3.4.5 Microbiota Composition of Quasi-Biofilms

A PCA plot was generated based on the results of the principal component analysis. The PCA was plotted to evaluate the similarity in the overall microbiota compositions of the quasi-biofilm samples by growth period and facility. As shown in Figure 3.4.6, the first two principal components (PCs) explain 26.61% of the variance in the compositional data. Of that, 17.28% of the variance is explained by the PC 1 and 9.33% of the variance is explained by the PC 2. There is clear clustering of samples by growth period (3-, 5-, and 15- day experiment), but no observed clustering by facility or treatment (Appendix Fig B.3.3.2).
Figure 3.4.6 PCA plot based on the centered log ratio OTU relative abundances in quasi-biofilm samples. Each symbol represents one sample. The samples are color-coded by growth period, where turquoise represents a 3-day assay (3D), green represents a 5-day assay (5D), and purple represents a 15-day (15D) assay quasi-biofilm microbiota composition with repeated culture addition. The square symbols indicate negative control (NC) samples, the plus symbol indicates positive control (PC) samples, the triangle symbol indicates samples with added PS01155, and the circle symbol indicates samples with added PS01156.

The relative abundance of bacterial taxa is shown in Figures 3.4.7-9 for samples from each facility. On average, each sample had 38.30 +/- 0.80% of their total identified composition made up of families that were less than 1% abundant. Families present in large relative abundance in all samples were unidentified families in the *Lactobacillaceae* (7.81 +/- 0.02%), *Gammaproteobacteria* (10.3 +/- 0.44%), and *Bacilli* (5.26 +/- 0.20%) Orders as well as Bacteria unclassified (5.11 +/- 0.20 %). The families *Enterobacteriaceae* (8.29 +/- 0.20%) and *Pseudomonadaceae* (4.32 +/- 0.27%) were also highly relative abundance in each sample (Figures 3.4.6-8).
Figure 3.4.7 The microbiota composition in quasi-biofilm samples grown from environmental microbiomes collected in F1. The circle size represents the relative abundance of bacterial families detected at the end of the experiments (3-, 5-, 15-days assays) in which environmental microbiomes collected from F1 were used. The largest point represents 30% relative abundance, and the smallest point represents 1% relative abundance. All bacterial families present in less than 1% relative abundance were grouped in the category “< 1% abund.”.
Figure 3.4.8 The microbiota composition in quasi-biofilm samples grown from environmental microbiomes collected in F2. The circle size represents the relative abundance of bacterial families detected at the end of the experiments (3-, 5-, 15-days assays) in which environmental microbiomes collected from F2 were used. The largest point represents 40% relative abundance, and the smallest point represents 1% relative abundance. All bacterial families present in less than 1% relative abundance were grouped in the category “< 1% abund.”.
Figure 3.4.9 The microbiota composition in quasi-biofilm samples grown from environmental microbiomes collected in F3. The circle size represents the relative abundance of bacterial families detected at the end of the experiments (3-, 5-, 15-days assays) in which environmental microbiomes collected from F3 were used. The largest point represents 30% relative abundance, and the smallest point represents 1% relative abundance. All bacterial families present in less than 1% relative abundance were grouped in the category “< 1% abund.”.

3.4.6 Differentially Abundant Taxa in Samples at 3-, 5-, and 15-days

PERMANOVA analysis determined that the microbiota composition of the samples from different facilities and different treatments (NC, PC, PS01155, PSO1156) were not significantly different from each other at the end of the experiments. However, the microbiota composition of the samples from different growth periods were found to be significantly different at the end of the experiment (Appendix Table B.3.1). Differential abundance calculations were performed
using ALDEx2 in order to determine the compositional differences among samples from 3-, 5-, and 15-day assays. Figure 3.3.10 displays all of the OTUs that were differentially abundant when comparing the three different growth periods to each other. Based on the differential abundance results, we concluded that the differences in relative abundances of those OTUs between the different biofilm end points were significant (Figure 3.4.10).
15-days assays) in all of the samples. The largest point represents 0.0002% relative abundance, and the smallest point represents 0.000001% relative abundance.

The 3-day quasi-biofilms had a higher relative abundance of ten bacterial families when compared to the 15-day biofilms. This includes multiple species of Moraxellaceae (OTUs 3, 46, 49), Rhodobacteraceae (OTUs 54, 59, 74, 107), Lactobacillaceae (OTUs 32, 36), Sphingomonadaceae (OTUs 62, 82, 87, 102), Beijerinckiaceae (OTUs 68, 97), and Rhizobiaceae (OTUs 20, 53). Additionally, the 3-day quasi-biofilms also had a higher relative abundance of species of Propionibacteriaceae (OTU 175), Microbacteriaceae (OTU 45), Caulobacteriaceae (OTU 40), and Micrococcaceae (OTU 38) (Figure 3.4.11B). However, when compared to the 5-day biofilms, the 3-day quasi-biofilms did not have any differentially abundant species identified. The 5-day biofilms had a higher relative abundance of species of Xanthomonadaceae (OTU 75), when compared to the 3-day quasi-biofilms. Five-day biofilms also had higher relative abundance of species from the families Lactobacillae (OTU 36), Micrococcaceae (OTU 38), and Enterobacteriaceae (OTU 9) (Figure 3.3.11C) when compared to the 15-day quasi-biofilms. Both the 3- and 5- day quasi- biofilms had a higher relative abundance of OTUs 36 and 38 when compared to the 15-day quasi-biofilms.

When compared to the 5- day quasi-biofilms, multiple species of the Pseudomonaceae (OTU 6 and 10) were found to be present in higher relative abundance in the 15-day quasi-biofilms, as well as species from the families Comamonadaceae (OTU 17), Shewanellaceae (OTU 24), Flavobacteriaceae (OTU 25), Alcaligenaceae (OTU 26), Rhizobiaceae (OTU 78), Xanthomonadaceae (OTU 13, 56), Dysgonomonadaceae (OTU 69), and Enterobacteriaceae (OTU 109) (Figure 3.3.11A). Additionally, the 15-day quasi-biofilms had a relative abundance of species from the families Carnobacteriaceae (OTU 12), Lactobacillaceae (OUT 32),
Morganellaceae (OTU 41), and Gammaproteobacteria unclassified (OTU 131, 174) when compared to the 3-day quasi-biofilms (Figure 3.3.10 B).

Figure 3.4.11 The differentially abundant taxa identified in 3-, 5-, and 15-day quasi-biofilms. The x axis represents the effect score calculated using the ALDEx2 for each OTU found to be differentially abundant. The color of the stacked bar plots represents the bacterial families that each OTU belongs to. The OTUs with effect sizes greater than 1 were considered significant.
3.5 Discussion

3.5.1 Putative Bacteriocins Produced by Strains PS01155 and PS01156

The strain PS01155 was identified as *Enterococcus faecium* and PS01156 was identified as *Enterococcus lactis* based on WGS data analysis, which differed from their identities reported by ATCC and Zhao, et al., 2004 (Zhao et al., 2004).

The web server BAGEL4 and BLAST analysis detected genes for enterocinB, enterocin SE-K4, enterolysin A and enterocin A in the genome of *E. faecium* strain PS01155. *Enterococcus* spp. typically produce more than one bacteriocin (Henning et al., 2015), which aligns with the detection of genes associated with the production of multiple bacteriocins in the genome of strain PS01155. Enterocin B, enterocin A and enterocin SE-K4 are class IIa bacteriocins, which is the most common class of bacteriocins produced by *Enterococcus* spp. and are known for their anti-listeria activity (Henning et al., 2015). *E. faecium* is the species that is most commonly reported as a producer of class IIa bacteriocins (Eguchi et al., 2001; Ness et al., 2014). Within class IIa, enterocin A has been identified as one of the most potent bacteriocins (Eijsink et al., 1998; Hickey et al., 2003; Nilsen et al., 1998). Strains that produce enterocin A, typically also produce enterocin B and other bacteriocins, which is in agreement with our genomic analyses results (Dündar et al., 2015; Strompfová et al., 2008). Enterolysin A is a class III bacteriocin which inactivates cells by degrading their cell wall structure leading to lysis but has not been shown to consistently inhibit *Listeria* spp. (Ness et al., 2014). The detection of bacteriocin-associated genes was consistent with the phenotypic results obtained using a spot-inoculation assay in which the strain PS01155 inhibited all tested *L. monocytogenes* strains. To evaluate whether the inhibition was due to bacteriocin production by PS01155, the putative
bacteriocins (i.e., inhibitory proteinaceous compounds) were partially isolated from supernatant of PS01155 when grown in MRS at both 24 and 48 hours. The inactivation of the antimicrobial activity by proteinase K suggests that the inhibition previously observed was due to substances that were proteinaceous in nature leading us to conclude that this inhibition is due to putative bacteriocins (E. Yang et al., 2018).

Analysis of the genome of PS01156 resulted in identification of genes associated with bacteriocins enterocin P, enterocin L50b, enterolysin A and UviB. Enterocin P and UviB are class IIa bacteriocin and enterocin L50b is a class II leaderless bacteriocin (L. M. Cintas et al., 1997; J. H. Kang & Lee, 2005; Nilsson et al., 2002). Enterocin P, UviB, and L50b have been reported to have antimicrobial activity against *L. monocytogenes* (Ness et al., 2014). Unlike the class IIa bacteriocins, leaderless bacteriocin secretion is not directed by an N terminal peptide, but most likely by ABC transporters, which means that the bacteriocins are active after translation. This is considered to be a simple secretion system, which is promising for commercial application (Perez et al., 2018).

Consistent with the detection of bacteriocin genes, the PS01156 pure culture inhibited all 22 tested *L. monocytogenes* strains when applied in the spot inoculation assay. However, at all tested growth temperatures, time and media combinations, other than growth in MRS at 6.2 pH for 48 hours, the filtered supernatant did not inhibit PS01273 *L. monocytogenes*. The supernatant of PS01156 grown in MRS at 6.2 pH for 48 hours exhibited only weak inhibition. Furthermore, once the hydrogen peroxide was removed, the inhibition was completely diminished. The results of the partial isolation of bacteriocins from PS01156 supernatant were inconsistent with previous reports of successful isolation of bacteriocins from the strain PS01156, previously reported as *E. durans* 152 (Du et al., 2017). The inability to produce supernatant with antimicrobial activity
also conflicted with the results from Cintas et al., 2000 (Luis M. Cintas et al., 2000) and remains unexplained.

3.5.2 Strains PS01155 and PS01156 Did Not Inhibit L. monocytogenes in 3- and 5-day Quasi-Biofilms

In the inhibition assay both LAB biocontrol strains inhibited all 22 L. monocytogenes isolate lawns. However, the strains did not significantly inhibit L. monocytogenes in the 3- and 5-day quasi-biofilms. In the 5-day quasi-biofilms L. monocytogenes reductions were all below 1 log CFU/mL, except for the F2 environmental microbiome samples co-cultured with the strain PS01155 where the concentration of L. monocytogenes slightly increased. In the 3-day quasi-biofilm samples the microbiome samples from F1 and F3 co-cultured with LAB reduced L. monocytogenes concentration from 1.30 to 2.19 log CFU/mL, but the reduction in the microbiome samples from F2 were less than 0.30 log CFU/mL. A potential reason for the difference in L. monocytogenes reduction among the microbiome samples collected from different facilities may be related to the initial level of naturally occurring L. monocytogenes in the environmental samples. F2 sample had a greater initial concentration of L. monocytogenes in (3.666 +/- 0.089 log CFU/mL) compared to F1 (not detectable) and F3 (1.586 +/- 0.359 log CFU/mL) (Appendix Table B.3.3). The LAB biocontrol strains may have been ineffective in significantly reducing L. monocytogenes in the 3- and 5-day quasi-biofilms because of competitive exclusion in the conditions used for growing quasi-biofilms. E. facieum and E. lactis are both mesophilic bacteria that may grow sub-optimally at 15°C. However, this temperature is favorable for the growth of other bacterial families that have been previously found in tree fruit packinghouses such as Flavobacteriaceae, Pseudomonadaceae, Moraxellaceae,
Xanthomonadaceae and Weeksellaceae (Tan et al., 2019). Furthermore, bacteriocin production by LAB is cell density dependent (Eijsink et al., 2002). The LAB strains were applied at high concentrations (1 x 10^7 CFU/mL) but slow growth and cell death due to competition with other microorganisms could potentially inhibit the production of bacteriocins as well as other secondary metabolites over time (Eijsink et al., 2002; Hibbing et al., 2010). Competitive exclusion may also be the reason the quasi-biofilms grown at each time period had significantly different microbiota compositions. Lastly, the experiment was designed to represent a worst-case scenario in which L. monocytogenes is present in a high concentration (1 x 10^5 CFU/mL), which was the maximum concentration observed in the previous study (Tan et al., 2019). It is possible that the LAB strains would be more effective if a lower concentration of L. monocytogenes were used; however, this remains to be tested.

The microbiota composition of each experimental end-point for all three growth periods (3-day, 5-day, and 15-day experiments) were significantly different from one another, as determined by PERMANOVA. Due to the small sample size, PERMANOVA was not performed to evaluate differences in microbiome composition among treatments within each growth period. The ALDEx2 results indicated that 3- and 5-day quasi-biofilms had less relatively abundant species from the families Pseudomonadaceae, Shewanellaceae, Flavobacteriaceae, Alcaligenaceae, and Comomadaceae when compared to the 15- day quasi-biofilms. Three-day quasi-biofilms had multiple species from the families Rhodobacteriaceae Sphingomonadaceae, Beijerinckiaceae, Rhizobiaceae, Moraxellaceae as well as single species from the families Propionibacteriaceae, Microbacteriaceae, Micrococcaceae, and Caulobacteriaceae that were present in a higher relative abundance compared to the 15-day quasi-biofilms. Five-day biofilms had a higher relative abundance of a single species from the bacterial families Lactobacillaceae,
Micrococcaceae and Enterobacteriaceae when compared to the 15-day biofilms. Many of the bacterial families that were present in higher relative abundance in the 3- and 5- day biofilms are composed of mesophilic bacteria that are commonly found in water, soil, air, and animals (Dastager et al., 2014; Glaeser & Kämpfer, 2014; Marín & Arahal, 2014; Pujalte et al., 2014; Teixeira & Merquior, 2014). The bacterial families that were present in a greater relative abundance in the 15-day biofilms contain multiple species with strong biofilm forming abilities, most notably, Pseudomonadaceae (Allison et al., 1998; Araújo et al., 2016; Auerbach et al., 2000; Meliani & Bensoltane, 2015; X. Pang & Yuk, 2019; Puga et al., 2018), Flavobacteriaceae (Basson et al., 2008; Bremer et al., 2001; Cai et al., 2013, 2019) and Shewanellaceae (Bagge et al., 2001; Cheng et al., 2020; Silva et al., 2020; Wu et al., 2013; Zhu et al., 2019). The families that were more abundant in the 3- and 5- day quasi-biofilms may have been less fit to compete with the bacteria that were found in a higher relative abundance in the 15-day biofilms. This may be due to the fact that many species of Pseudomonadaceae are able to dominate biofilms (Heir et al., 2018; Y. Liu et al., 2016; Røder et al., 2015; Stellato et al., 2017). Both Pseudomonadaceae and Flavobacteriaceae have been shown to enhance L. monocytogenes growth in food processing facility biofilms (Bremer et al., 2001; Hassan et al., 2004; Jeong & Frank, 1994; Liu et al., 2016; Puga et al., 2018). Additionally, many species of Pseudomonadaceae (Arslan et al., 2011; Caldera et al., 2016; Dogan & Boor, 2003; Rajmohan et al., 2002; Raposo et al., 2016), Flavobacteriaceae (Everton et al., 1968), and Shewanellaceae (Betts, 2006; Ge et al., 2017; Gram & Melchior, 1996; Korber et al., 2009; Qian et al., 2018) are spoilage organisms, which should also be targeted in cleaning and sanitizing routines in order to ensure food quality. The successful growth of these families in quasi-biofilms may be of concern for facilities trying to reduce spoilage bacteria in their environment.
3.5.3 Repeated Biocontrol Strain Application in 15-day Quasi-Biofilms Reduced L. monocytogenes Concentration

The 15-day quasi-biofilms with repeated LAB biocontrol application was performed in order to assess the additive effect of the biocontrol strains on the L. monocytogenes concentration in a quasi-biofilm on day 15 after first application. The reduction of L. monocytogenes in the quasi-biofilms with LAB strains was statistically significant (p=0.00179), but practically insignificant (less than 0.50 log CFU/mL) as L. monocytogenes has been found at concentrations of 5 log MPN/mL in tree fruit packinghouses (Tan et al., 2019). In two studies that had applied the same LAB biocontrol strains in two different poultry processing facility drains, the strains were applied after cleaning and sanitizing four days in a row for the first week. For the next three weeks, they applied the strains twice a week, and then continued to sample for up to 18 weeks after the last treatment (Zhao et al., 2006, 2013). In one study modest Listeria spp. inhibition was observed in the first two weeks; however, the inhibition reached 4.1 log CFU/mL at the end of the study (Zhao et al., 2006). In the second ready-to eat poultry facility’s drains, they found that the Listeria spp. were undetectable in five of the six drains tested after the first week (Zhao et al., 2013). In our study, biofilms were “cleaned” with 1 x PBS and the two LAB strains were reapplied on the fifth and tenth day of growth. This interval was chosen because there was no change in the L. monocytogenes concentration in the five-day quasi-biofilms and we wanted to examine if the treatment would be effective with repeated application of the biocontrol strains. The results from their studies may mean that the LAB biocontrol strains need to be regularly introduced to the microbiome in order for them to be effective in reducing Listeria spp. due to competition. Furthermore, the strains were added together to all drains, indicating that the
addition of both strains might have an increased ability to compete with and inhibit *Listeria* spp., compared to the addition of just one of the strains to each microbiome sample in our study. In the paper of Zhao et al. (2013), only one of the biocontrol strains were found in the biofilms at the end of 8 weeks at a concentration of 100 CFU/cm². The strains had originally been applied at a concentration of $1 \times 10^7$ CFU/mL, suggesting that the LAB strains did not thrive in the environment. These results from Zhao et al. (2006 and 2013) as well as the results of our study suggest that the biocontrol strain addition to the environment would need to be part of a daily sanitation routine in order to be effective. Furthermore, the differences in Zhao et al. and our results suggest that the environmental microbiota composition as well as the concentration of *L. monocytogenes* may affect the efficacy of the biocontrol; however, it is important to note that our experimental design did not allow us to measure the effect of these variables on the efficacy of LAB in reducing *L. monocytogenes* in a quasi-biofilm.

### 3.5.4 Future Work: Focus on Biofilm Control

This study has shown that assessing the ability of biocontrol strains to inhibit pure cultures of *L. monocytogenes* is not indicative of their inhibition of *L. monocytogenes* when cocultured with a food processing environmental microbiome. Biocontrol strains and many bacteriocins have a very specific spectrum of antimicrobial activity (Henning et al., 2015; Simons et al., 2020). Future studies evaluating the efficacy of biocontrol strains should therefore not only test their efficacy against pure cultures of the target pathogen, but also against the pathogen in the microbiome that resembles the target environment in which biocontrols are intended to be used. This would allow for the assessment of the feasibility of successfully applying biocontrol strains in food industry.
Control strategies of *L. monocytogenes* in commercial food processing environments should not be dependent on the existing microbiota in a facility and should be effective regardless of the background microbiota. Strains PS01155 and PS01156 may not be the solution for enhancing the control of *L. monocytogenes* in tree fruit packinghouses based on the results of this study.

This study did show that *L. monocytogenes* can grow up to 2 log CFU/mL when grown for 15-days with tree fruit packinghouse microbiota into a quasi-biofilm. This demonstrates a need to enhance the control of *L. monocytogenes* in tree fruit packinghouse environments by effectively removing biofilms. Without enhancements in cleaning and sanitizing, *L. monocytogenes* may colonize and thrive in these food processing environments. Future studies should therefore focus on the evaluation of best practices for the control of biofilms in tree fruit packinghouse environments, including the use of disinfectant treatments designed to dissolve biofilm structure.

### 3.5.5 Limitations

This study has several limitations, we only tested the two biocontrol strains against 22 *L. monocytogenes* strains and there may be others that are not impacted by PS01155 and PS01156. Additionally, we were unable to partially isolate bacteriocins from PS01156, which did not agree with the BAGEL 4.0 results and other studies that have successfully isolated bacteriocins from that strain. The small standard deviation in the 15-day quasi-biofilms may have led to the slight differences in the *L. monocytogenes* concentration being considered significant leading to a potential false positive.
Although, next generation sequencing allows for a deeper understanding of microbiomes, however it is not fully representative of the entire microbiota as it is not able to account for total microorganisms in an environment. Illumina sequencing can only detect a finite amount of organisms (G. B. Gloor et al., 2017). The relative abundance of *Listeriaceae* in the results of the microbiome analysis displays the lack of sensitivity of amplicon sequencing for the detection of species that are present in low abundance. Although *L. monocytogenes* is at a relatively high concentration in some samples (ranging from 2.4 to 7 log CFU/mL), the *Listeriaceae* family is less than 1% relatively abundant in each sample microbiota. This may mean the bacterial families that drove the difference in the microbiota compositions at each growth period may be due to low-abundant bacteria that have not been detected by amplicon sequencing. This could also explain a highly similar composition of microbiota at the end point of each growth period experiment.
Chapter 4

Conclusion and Future Directions

These studies have evaluated the difference in the microbiota of animal- and plant-based food processing environments as well as the ability of biocontrol strains to inhibit *L. monocytogenes* when grown in quasi-biofilms with tree fruit packinghouse microbiota. The overall shared common core microbiota as well as the identified animal- and plant-based common core microbiota indicate that the most prevalent bacteria have biofilm forming potential and grow at low temperatures. The microbiota of plant and animal-based food processing environments were found to be different. In the common core microbiota of plant- and animal-based food processing environments, there were multiple families with species that are known to enhance biofilm formation, the growth of *L. monocytogenes* and other pathogens. This emphasizes the need for cleaning and sanitizing routines to target not only pathogens but also biofilm removal in all food processing environments. Development of cleaning and sanitizing procedural and disinfectant enhancements should be pursued for biofilm removal.

*L. monocytogenes* can form an attached biomass (quasi-biofilms) with the microbiome of tree fruit packinghouses at 15°C. The two biocontrol strains PS01155 and PS01156 were unable to reduce *L. monocytogenes* in the 3- and 5-day quasi-biofilms. However, the biocontrol strains were both able to reduce *L. monocytogenes* when repeatedly applied over 15-days. Although the reductions were found to be statistically significant, they were not practically significant. The microflora that exists in tree fruit packinghouses may influence the ability of *L. monocytogenes* to survive in the environment. Multiple bacterial families that were found in the microbiota analysis are known to form biofilms and potentially enhance the growth of *L.*
monocytogenes when cultured together. L. monocytogenes was inhibited by both strains in the spot inoculation assay, which indicates that biocontrol efficiency should be tested both against the foodborne pathogen itself and in the microflora in which its application is desired.

Further steps for this thesis should be taken by assessing different cleaning and sanitizing procedure’s ability to reduce L. monocytogenes before and after cleaning and sanitizing. Additionally, there should be more research into different applications of biocontrol strains to test their efficacy, including both increased frequency of application and in different environments. Future work should also focus on controlling pathogens within biofilms as well as biofilm removers as they act as protective shield for the microorganisms within them. Focus on products that are able to dissolve the EPS.
Appendix A

Effect of Different Cleaning and Sanitizing Protocols on Prevalence and Levels
Listeria monocytogenes and Aerobic Mesophilic Bacteria in Tree fruit Packing Environments

A.1 Introduction

*L. monocytogenes* is a foodborne pathogen that is one of the leading causes of foodborne related deaths in the U.S.A (CDC, 2018). *L. monocytogenes* is widespread in the food processing environment due to its ability to survive and grow in continuously wet conditions at low temperatures (Azizoglu et al., 2009) in addition to its ability to form protective biofilms (Alavi & Hansen, 2013; Colagiorgi et al., 2017). Despite the widespread availability of industry guidelines for best cleaning and sanitizing procedures, *L. monocytogenes* has caused several recalls and outbreaks, including a multi-state outbreak of contaminated caramel coated apples that caused 34 hospitalizations and 7 deaths (FDA, 2015). This, and other *Listeria* related incidents in tree fruit packinghouses have stimulated an increase in studies to monitor *L. monocytogenes* in these facilities.

In a 3-year study of 3 northeastern U.S. tree fruit facilities conducted in 2016-2019, the overall occurrence of *L. monocytogenes* was 17.5%, with the highest occurrence in the packing line area (19.7%) followed by cold storage rooms (16.4%) and the packaging area (6.4%) (Simonetti, et al., 2021). In a one-year study conducted in 2017-2018 at the same packing facilities, *L. monocytogenes* was detected at zone 3 sites (underneath the packing line) in all facilities at varying frequencies, including one facility where it was detected in all samples taken (Tan et al., 2019). In the southeastern U.S., eleven packinghouses were sampled for *L.*
monocytogenes, two of which packed tree fruit. The study found that L. monocytogenes, Listeria spp., and both L. monocytogenes and Listeria spp. were present in 3.2%, 2.7%, and 0.6% of samples respectively (Estrada et al., 2020). Between 2018 and 2020 five commercial apple packinghouses were sampled for Listeria spp. in the pacific northwest, finding an overall occurrence in the samples of 4.6% (Ruiz-Llacsahuanga et al., 2021). In this study, the highest occurring sample locations were the wax coating areas, polishing brushes, dividers and brushes under fans, and dry rollers. Transient Listeria spp. in a packinghouse is not necessarily dangerous, however, when Listeria spp. and L. monocytogenes become persistent in a facility, they are more likely to contaminate the product and spread throughout the facility (UFPA, 2018).

Persistent L. monocytogenes was observed by Tan, et al, 2019 and Simonetti et al, 2021, especially at sample locations around the packing lines of the facilities. A further investigation by Chen, et al, 2021, using whole genome sequencing of the L. monocytogenes isolates, showed that in the facility with the highest occurrence of L. monocytogenes, the isolates were all closely related, suggesting that one strain was predominant in the facility (Chen et al.).

The results from these studies suggest that the cleaning and sanitizing practices currently employed in tree fruit packinghouses monitored by Tan et al. and Simonetti et al. may be insufficient for controlling and eliminating L. monocytogenes from the environment. The aim of this study was therefore to evaluate the effectiveness of “industry best practices” sanitation standard operating procedures (SSOP) for reducing aerobic mesophilic microorganisms and L. monocytogenes in the three (F1, F2, F3) tree fruit packinghouse environment; specifically, in areas around the packing line.
### A.2 Methods

#### A.2.1 SSOP Development and Treatments

The four SSOP treatments used in this study were developed with the consultation of industry experts and using the United Fresh guidelines for packinghouse *Listeria* control (UFPA, 2018). The first SSOP in each of the facilities was their baseline procedure (T1) (Table A.2.1). The second procedure (T2) included the use of power washing to remove debris from roller brushes (Picture A.1), a wax remover on the wax area, chlorinated alkaline foam cleaner with scrubbing for 20 minutes on all equipment, followed by the application of Peracetic acid (PAA) (Appendix B.3.3). The third treatment (T3) was the same as the second with the addition of a sulfuric acid cleaner on all stainless-steel surfaces (Appendix B.3.4). The fourth treatment (T4) was the same as the second treatment except that Sterilex™, a disinfectant targeted at breaking up biofilms was applied (Appendix B.3.5). The SSOPs were performed in three different packinghouses. Each treatment was applied once for this preliminary study, which also served to train the cleaning and sanitizing crews on the implementation of three new SSOPs. Five locations were sampled in each facility.
Table A.2.1. Cleaners and sanitizers used in three participating apple packinghouses prior to the study.

<table>
<thead>
<tr>
<th>Facility</th>
<th>Cleaner</th>
<th>Sanitizer</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>Potable water</td>
<td>ChemSanHQ</td>
</tr>
<tr>
<td>F2</td>
<td>Inspectors Choice and Wax Strip</td>
<td>Sani-T-10</td>
</tr>
<tr>
<td>F3</td>
<td>Mixture of Dawn Detergent and bleach</td>
<td>NA</td>
</tr>
</tbody>
</table>

After the completion of the supervised application of the baseline SSOPs (T1) and three different treatment SSOPs (T2, T3, T4), long-term application of a combination of T2 and T4 was carried out in one facility (F2) without supervision of the research team. First, T2 was applied every weekday for four weeks starting on a Wednesday. On the following Wednesday morning samples were collected in the same five locations as in the initial evaluation of four treatments. These samples were tested to quantify aerobic mesophilic bacteria and *L. monocytogenes*, the results of which were used to evaluate the effectiveness of a long-term application of prescribed sanitizing protocol. After the initial four weeks of applying T2 on a weekly basis, the T2 was continued to be applied every day of the week, except for Friday, for the next four weeks. On Friday, T4 was applied (on a weekly basis) to control biofilm formation. Samples were on the following Wednesday morning prior to the start of tree fruit packing, to assess the impact of weekly application of T4 and daily application of T2. Samples were tested to quantify aerobic mesophilic bacteria and *L. monocytogenes*. 
A.2.3 Sampling Plan

All four SSOPs (T1, T2, T3, and T4) were tested in three different facilities; each treatment was tested once in each of the three facilities. Samples were collected for microbiota characterization, quantification of aerobic mesophilic microorganisms and *L. monocytogenes* before cleaning and sanitizing began and after cleaning and sanitizing, in the morning before packing in order to allow the sanitizer to dry overnight. Microbiome samples were taken with 3M Hydrated Sponge, 1.5” x 3” Sponge w/10mL Letheen Broth, *L. monocytogenes* enrichment and enumeration samples were taken with 3M Hydrated Sponge, 1.5” x 3” Sponge w/10mL D/E
Neutralizing Buffer. Samples were taken in five locations, in zone 2, around the apple packing line (Table A.2). The implementation of SSOPs were monitored by the research team, the research team was available to answer any questions from the sanitation crew. For treatment 4, the technical sales representatives from Sterilex were also present on site. Samples were stored on ice and processed in the next 48 hours.

A.2.2. Sample labels and locations

<table>
<thead>
<tr>
<th>Code</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>Floor crack</td>
</tr>
<tr>
<td>S2</td>
<td>Under washing area</td>
</tr>
<tr>
<td>S3</td>
<td>Under drying area</td>
</tr>
<tr>
<td>S4</td>
<td>Under waxing area</td>
</tr>
<tr>
<td>S5</td>
<td>Leg-floor connection by waxing area</td>
</tr>
</tbody>
</table>

A.2.4 Processing of Samples for Microbiome Characterization and Quantification of Aerobic Mesophilic Microorganisms

The samples taken with 3M Hydrated Sponge, 1.5" x 3" Sponge w/10mL Letheen Broth were processed by adding 40 mL of BHI to each sample bag. Each sample was stomached for 7 minutes at 230 rpms. Two milliliters of each sample were then used for aerobic plate counts. The rest of the samples were then centrifuged for 20 minutes at 11,000 g and 4 °C. The resulting supernatant was discarded, and the pellets were kept at -80°C for downstream microbiome analysis.
Two mL of each sample were used for an initial 1:2 dilution followed by a series of 1:10 dilutions. Each dilution was plated in duplicate on aerobic Petrifilms (3M, Minnesota) and incubated at 35°C for 48 hours. After 48 hours, Petrifilms with 15-150 CFU were counted. The significance of APC reductions for each assay was assessed by performing a paired t-test and test using a package stats v. 4.0.3 in R v. 3.6.1 (R Core Team, 2020). Standard error was calculated for technical replicates from each independent experiment based on the standard deviation in R v. 3.6.1.

A.2.5 Enrichment and Quantification of Listeria and Listeria monocytogenes

*L. monocytogenes* was enriched and quantified according to the Food and Drug administration Bacteriological Analytical Manual (BAM) Detection and Enumeration of *Listeria monocytogenes* (FDA, 2019c) and BAM Most Probable Number from Serial Dilutions (FDA, 2019). Samples for *L. monocytogenes* enrichment and enumeration were taken with 3M™ Hydrated Sponge, 1.5" x 3" Sponge w/10mL D/E Neutralizing Buffer. For *L. monocytogenes* enrichment, 90 mL of BLEB were added to each sample bag and massaged for 30 seconds. A positive control was made by adding a single colony of PS00838 to a sample sponge and then adding 90 mL of BLEB and massaging the bag for 30 seconds. A negative control was made by adding 90 mL of BLEB to an unused sample bag. The sample bags were then incubated for 4 hours at 30°C. After 4 hours of incubation, 400 µl of BLEB supplement (10 mg/L acriflavin, 50 mg/L nalidixic acid, and 40 mg/L cycloheximide; Sigma Aldrich, St Louis, MO), was added to each sample and the tubes were further incubated at 30 °C for additional 44 +/− 2 hours. After incubation, one loopful from each sample was swabbed onto an Agar *Listeria* Ottavani & Agosti (ALOA) agar plates (BioRad, Marnes la Coquette, France) and a RAPID plate. Inoculated plates
were incubated for 24 and 48 hours at 37°C. After incubation, one perspective colony of *L. monocytogenes* from ALOA (blue/green with a halo) and/or RapidLmono (blue colonies) were struck for isolation on BHIA. *L. monocytogenes* PS00838 and *L. innocua* PS00298 were used as positive controls. One colony from each BHI plate was then grown overnight in BHI. One milliliter of each culture was then centrifuged for 15 minutes at 15,000 g. The resulting pellets were then resuspended in 100 µl of DNase free water and then heated to 95°C for 15 minutes. Samples were then cooled for 5 minutes and centrifuged for 10 minutes at 15,000g. In order to confirm that these colonies are *L. monocytogenes*, the gene *lmo2234* was then amplified to confirm their identification of *L. monocytogenes* and *iap* was amplified to confirm that the isolates were *Listeria* spp.

For *L. monocytogenes* and *Listeria* spp. enumeration, 100 µl of each dilution were inoculated into three microcentrifuge tubes pre-filled with 900 µl of buffered enrichment broth (BLEB) *Listeria* (Hardy Diagnostics, Santa Maria, CA). Inoculated microcentrifuge tubes were incubated at 30°C for 4 hours. After 4 hours of incubation, 4 µl of BLEB supplement (Sigma Aldrich, St Louis, MO), was added to each sample and the tubes were further incubated at 30 °C for additional 44 +/- 2 hours. After incubation, one loopful of each MPN dilution (from $1 \times 10^{-2}$ through $10^{-9}$) was streaked in triplicates onto ALOA agar plates (BioRad, Marnes la Coquette, France). Inoculated plates were incubated for 24 and 48 hours at 37 °C. After 24 and 48-hour incubation, plates were examined for growth of blue-green colonies with a halo. Each dilution replicate that had blue-green colonies with a halo was considered positive for *L. monocytogenes* growth. The BAM MPN calculator downloaded from the FDA BAM website (FDA, 2019) was used to calculate the MPN/tube. The limit of quantification (LOD) of *L. monocytogenes* and *Listeria* spp. was 100 log CFU/swab. The significance of *L. monocytogenes* reduction for each
treatment was assessed by performing a paired t test using a package stats v. 4.0.3 in R v. 3.6.1 (R Core Team, 2020). Standard error was calculated for technical replicates from each independent experiment based on the standard deviation in R v. 3.6.1.

**Results**

*Facility application*

In facility 1 (F1), the treatment was applied by a second shift crew, and was primarily performed by two people, on February 9th and 23rd, March 2nd and 9th 2021. The cleaning crew started the process at 5 pm and ended at 12 am. In facility 2 (F2), the SSOPs were applied by a single, second shift worker on November 9th and 30th, December 7th and 14th 2020. The cleaning individual started the process at 4 pm and ended at 9 pm. In facility 3 (F3), the SSOPs were applied by a crew of six members that worked in the packinghouse during the day, after packing concluded, on January 19th, 27th and February 2nd and 16th 2021. The cleaning crew started the process at 4 pm and ended at 6 pm. The facility baseline SSOPs differed in each facility and the products used are included in Table B.3.1. In addition to the one-time application of T1, T2, T3, and T4, a month-long application of T2 (preceded by one application of T4) was carried out in F2 (January 19th to February 17th). Subsequent to the first month-long application of T2, another month-long application of T2 was carried out, only this time T4 was applied once a week (February 19th to March 17th).
<table>
<thead>
<tr>
<th>Sanitation Step</th>
<th>Chemical Type</th>
<th>Product Name and Supplier</th>
<th>Application Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cleaner</td>
<td>Chlorinated alkaline foam</td>
<td>AC36 Concentrate. (Arcadia Chemical Inc.)</td>
<td>1:14.3 v/v</td>
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<td>Acid Cleaner</td>
<td>GreenClean Acid Cleaner (BioSafe Systems Inc.)</td>
<td>1:32 v/v</td>
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<td>De-waxer</td>
<td>Wax Strip Plus (Pace Intern. Inc)</td>
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<td>Sanitizer</td>
<td>Peroxyacetic acid + Hydrogen peroxide</td>
<td>SaniDate 5.0 Concentrate (BioSafe Systems Inc.)</td>
<td>1:256 v/v (230 ppm PAA)</td>
</tr>
<tr>
<td>Disinfectant</td>
<td>PerQuat®</td>
<td>Sterilex Ultra Disinfectant, shelf life 18 months, and Sterilex Ultra Disinfectant Activator, shelf life 24 months (Sterilex Inc)</td>
<td>1:1:8 – 1:1:10 (12.8 – 16 oz/g)</td>
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<td>THU</td>
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<td>-----------</td>
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</tr>
<tr>
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<td>Sample collection</td>
<td></td>
</tr>
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Figure A.2.1: Calendar of the first long term treatment month. Treatment 4 (T4) was applied once and then treatment 2 (T2) was applied daily for a month. Samples were collected in the morning, before packing began.
<table>
<thead>
<tr>
<th>MON</th>
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<th>THU</th>
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<td>Feb-19 T4</td>
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<td>Feb-26 T4</td>
</tr>
<tr>
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</tr>
<tr>
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<td>Sample Collection</td>
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</tr>
<tr>
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<td>Mar-4</td>
<td>Mar-5 T4</td>
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<td>T2</td>
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</tr>
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<td>T2</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>Sample Collection</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure A.2.2: Calendar of the first long term treatment month. Treatment 4 (T4) was applied weekly on Friday and treatment 2 (T2) was applied Monday to Thursday for a month. Samples were collected in the morning, before packing began.

**Reduction in Aerobic Mesophilic Microorganisms**

The facility baseline SSOP (T1) in F2 and F3 did not reduce the overall aerobic plate count (APC). Facility 3’s SSOP resulted in a significant mean reduction of APCs by 1.61 log CFU/swab ($p=0.0014$). Treatment 3 was the only SSOP that significantly reduced APCs in all facilities. Mean reduction of APCs were 1.63 log CFU/swab ($p=0.0005$), 1.13 log CFU/swab ($p=0.0029$), and 2.23 log CFU/swab ($p=0.0059$) in F1, F2 and F3, respectively. Treatment 2 significantly reduced F2 APCs, the mean reduction of APCs was 1.38 log CFU/swab ($p=0.01633$). The application of T4 significantly reduced total APCs only in F3, where the average APCs reduction was 2.26 log CFU/swab ($p=0.00019$).
Figure A.3.1 Aerobic plate counts in samples collected before and after four different cleaning and sanitizing procedures (T1-T4) taken in three facilities (F1-F3). The green bars represent the average log CFU/swab of APC before cleaning and sanitizing and the yellow bars represent the average log CFU/swab of APC after cleaning and sanitizing. All bars include standard error bars.

In the first month-long study, the first application of T2 resulted in a larger reduction of APCs compared to all subsequent applications of T2, as well as compared to all subsequent daily applications of T2 and weekly applications of T4 in the second month-long study. The “After” APCs did not change from week to week, after the treatments were applied over a month.
Figure A.3.2 Long Term Sampling APC Counts in Facility 2. The average aerobic plate counts (APC) after cleaning and sanitizing are reported for each week just T2 was applied daily (graph label “T2”) or T2 was applied daily and T4 was applied weekly (graph label “T2+T4”). For “T2”, SSOP treatment 2 was applied every week-day for four weeks. For “T2+T4”, treatment 2 was applied Monday through Thursday and treatment 4 was applied on Friday, for 4 weeks. Each bar with standard error bars represents the average APC log CFU/swab after cleaning and sanitizing each week.

**Reduction in L. monocytogenes and Listeria spp. concentration**

Listeria spp. were only enumerated in F1 and F3. Listeria spp. was only significantly reduced in F3 by treatments 3, which resulted in a mean reduction of 3.039 log MPN/swab (p=0.008). L. monocytogenes was not above the limit of quantification in many of the samples, as shown by the large standard error in Figure A.3.4. If the enriched samples were positive for L. monocytogenes, plus signs were added above the average log MPN/mL bars in Figure A.3.4. The
number of *L. monocytogenes* positive samples decreased in F1 and F2 to zero after T4. However, in F3 *L. monocytogenes* positive samples was reduced most after T3, bringing the number of positive samples from 2 to 1.

Figure A.3.3 MPN of *Listeria* spp. in samples collected before and after four different cleaning and sanitizing treatments (T1-T4) from F1 and F3. The purple bars represent the average log MPN/swab of *Listeria* spp. before cleaning and sanitizing and the blue bars represent the average log MPN/swab of *Listeria* spp. after cleaning and sanitizing. All bars include standard error bars.
Figure A.3.4 MPN of *L. monocytogenes* in samples collected before and after four different cleaning and sanitizing procedures (T1-T4) from F1, F2, and F3. The pink bars represent the average log MPN/swab of *L. monocytogenes* before cleaning and sanitizing and the yellow bars represent the average log MPN/swab of *L. monocytogenes* after cleaning and sanitizing, all bars included standard error bars. The positive marks above each bar indicate if any of the samples collected at that time were positive for *L. monocytogenes* enrichment.

In the long-term study, *L. monocytogenes* concentrations were below the limit of detection after treatment 4 was applied weekly and treatment 2 daily, compared to only treatment 2 being applied daily. *Listeria* spp. did not significantly change over time during either treatment (p=0.869, p=0.0962). Over the 8 weeks, one sampled area, the floor under the wax area, was positive for *L. monocytogenes* each week when sample was collected from this area. In the long-term study, the cleaning and sanitizing was unsupervised by the research team.
Figure A.3.5 Long-term Sampling *Listeria* spp. Counts in F2. The average *Listeria* spp. after cleaning and sanitizing are reported for each week T2 or T2 and T4 were applied. For T2, SSOP treatment 2 was applied every weekday for four weeks. For T2+T4, treatment 2 was applied Monday-Thursday and treatment 4 was applied on Friday, for 4 weeks. Each bar represents the average *Listeria* spp. log MPN/swab after cleaning or sanitizing each week with standard error bars.
Figure A.3.6 Long Term Sampling *L. monocytogenes* counts in F2. The average *L. monocytogenes* after cleaning and sanitizing are reported for each week T2 or T4 were applied. For T2, SSOP T2 was applied every weekday for four weeks. For T2+T4, T2 was applied Monday-Thursday and T4 was applied on Friday, for 4 weeks. Each bar represents the average *L. monocytogenes* log MPN/swab after cleaning or sanitizing each week with standard error bars.

**Discussion**

Overall APC reductions were all less than 3 logs CFU/swab. The only treatment that significantly reduced APCs in all facilities was T3, however, T3 did not reduce *Listeria* spp. in F1 or F2 or eliminate *L. monocytogenes* from the samples. In both F1 and F2 treatment 4 eliminated *L. monocytogenes* from the samples. This was not the case in F3, the most effective treatment for reducing *L. monocytogenes* in the samples was T3. Treatment 3 may have been more effective at reducing *Listeria* spp., APC, and *L. monocytogenes* positive samples in F3, while not in the other facilities because F3 had stainless steel pans below their packing line that
were close to the floor, the acid treatment was only applied to stainless steel. One possibility for this difference is that the dissolution of the biofilm structure may not have only freed all of the bacteria but also organic matter that may have inactivated the sanitizer despite rinsing (Lone Gram et al., 2007; Korber et al., 2009).

The *L. monocytogenes* enrichment results from the long-term study had only one sampled location positive for *L. monocytogenes* over the 8 weeks of weekly sampling. This location was directly under the wax section of the packing line. Our results align with the results from Ruiz-Llacshahuanga, et. al, 2021, which sampled five packinghouse for *Listeria* spp. finding one of the highest occurrence of *Listeria* spp. in the wax coating area (Ruiz-Llacshahuanga et al., 2021). The consistent occurrence of *L. monocytogenes* under the wax area may suggest that the wax is not being fully removed using these treatments and/or the wax remover may need to be applied for a longer time. Macarisin, et al. (2019), showed that the wax coating can help *L. monocytogenes* survive and protect it from desiccation (Macarisin et al., 2019). However other foodborne pathogens, such as *E. coli* O15:H7 and *Salmonella* Muenchen’s survival on apples was impaired by wax coatings, resulting in up to 1.48 log CFU/ apple reductions (Kenney & Beuchat, 2002). A possible reason for this may be the fact that Gram negative bacteria are more susceptible to isopropanol, which is present in wax, than gram positive bacteria (Macarisin et al., 2019). These results suggest that the use of a disinfectant that is designed to degrade biofilm structure is necessary to remove *L. monocytogenes* in tree fruit packinghouses. Additionally, the results in the long-term study suggest that the development of enhancements for wax removal may help to eradicate entrenched *L. monocytogenes* from tree fruit packinghouse environments.

ATP swabs were taken before and after cleaning (not sanitizing). The results were variable and there was no correlation between values before or after cleaning, so this was not
included in the results. We do not recommend the use of ATP in tracking the levels of cleanliness overtime.

Possible future directions for this study would be to assess the efficacy of a longer contact time of the wax remover or the investigation of different commercially available wax removers. Additionally, the long-term study should be performed in the other two facilities in order to assess the impact of the T2 and T2 plus T4 in all facilities. There were many factors in this study that could not be controlled given that the evaluation of treatment effectiveness was carried out in the actual packing facilities. For example, facilities had different sizes of cleaning crews, different line size, age of packing line, sanitary design of equipment, baseline SSOPs, supervision, food safety expertise, and equipment, which may have influenced the results of this study. Additionally, in the long-term study, the researchers were not supervising cleaning and sanitizing which may have led to the difference in results after T4 in the one-time application trial prior to subsequent daily application of T2 and T4 in weekly application trial.
Appendix B

Table B.3.1 PERMANOVA Results for the Microbiota of Meat vs Dairy vs Plant Based Pairs

<table>
<thead>
<tr>
<th>Pairs</th>
<th>Df</th>
<th>SumsOfSqs</th>
<th>F.Model</th>
<th>R2</th>
<th>p.value</th>
<th>p.adjusted</th>
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</thead>
<tbody>
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<td>Dairy vs Plant</td>
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<td>104833.6</td>
<td>159.8254</td>
<td>0.2267588</td>
<td>0.001</td>
<td>0.003</td>
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<td>295079.1</td>
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<td>0.3806468</td>
<td>0.001</td>
<td>0.003</td>
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<tr>
<td>Plant vs Meat</td>
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<td>131229.3</td>
<td>151.0293</td>
<td>0.2005623</td>
<td>0.001</td>
<td>0.003</td>
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</table>

Figure B.3.2 Clustering of samples based on PCA analysis of bacterial families. Samples are color-coded based on the region of the 16s rRNA gene that was amplified. Squares denote samples originating from plant-based facilities, triangles denote samples originating from dairy-based food processing facilities, and circles denote samples originating from meat-based food processing facilities.
Figure B.2.1 Phylogenetic Tree of whole genome sequenced isolates collected in Tree fruit packinghouses over a three-year period. Twenty-two isolates that represent 22 out of the 23 clades on this tree were selected to be tested in the spot inoculation assay by CFSAN scientist Yi Chen. One clade was not represented because its single isolate was not in stock.
Figure B 3.3.1 Section of ML tree built in Ape v. 5.4.1. The LAB strains are indicated as “ED A”, “ED ATCC new stock”, “LL A”, “LL ATCC” new Stock. The LAB cultures were found to be closely related to each other as well as *E. lactis* and *E. facieum*, the concluded identity of the strains based on whole genome sequencing.
Figure B.3.2 PCA of all quasi-biofilm samples. The samples are colored by Facility, blue representing F2, green representing F3, and purple representing F1 samples. The samples are shaped by whether they were the negative control (NC), positive control (PC), PS01155 (LL), PS01156 (ED).

Table B.3.2 PERMANOVA Results for Quasi-Biofilm Experiment

<table>
<thead>
<tr>
<th>Condition</th>
<th>Pairs</th>
<th>Df</th>
<th>SumsOfSqs</th>
<th>F.Model</th>
<th>R2</th>
<th>p.value</th>
<th>p.adjusted</th>
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<td>Growth Period</td>
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<td>5D vs 3D</td>
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<td>1</td>
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<td>5D vs 15D</td>
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<td>0.003</td>
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<td>1</td>
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<td>0.003</td>
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<td>Facility</td>
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<td>F1 vs F3</td>
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<td>1</td>
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<td>1.001778</td>
<td>0.0154116</td>
<td>0.402</td>
<td>1</td>
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<tr>
<td>F1 vs F2</td>
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<td>1</td>
<td>1883.204</td>
<td>1.527317</td>
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<td>0.195</td>
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<td>F3 vs F2</td>
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<td>1306.716</td>
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Table B.3.3. Tukey’s HSD results for the PS01156 results in the spot inoculation assay.

<table>
<thead>
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<th>Concentration (CFU/mL)</th>
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<th>p-value</th>
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<tr>
<td></td>
<td>15-30</td>
<td>0.0000045</td>
</tr>
<tr>
<td></td>
<td>20-25</td>
<td>0.9881018</td>
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<tr>
<td></td>
<td>15-25</td>
<td>0.0036668</td>
</tr>
<tr>
<td></td>
<td>15-20</td>
<td>0.0103862</td>
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<tr>
<td>10⁸</td>
<td>25-30</td>
<td>0.0354233</td>
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<tr>
<td></td>
<td>20-30</td>
<td>0.0008115</td>
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<td></td>
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<td>20-25</td>
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<tr>
<td></td>
<td>15-20</td>
<td>0.7108070</td>
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*These results display whether the inhibition observed at each temperature was significantly different from one another at both concentrations.

Table B.3.4 The APC and *L. monocytogenes* concentrations in the starting Facility samples used for quasi biofilms

<table>
<thead>
<tr>
<th>Facility</th>
<th>APC log CFU/mL</th>
<th><em>L. monocytogenes</em> log MPN/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>F1</td>
<td>6.321 +/- 0.478</td>
<td>ND*</td>
</tr>
<tr>
<td>F2</td>
<td>5.404 +/- 0.261</td>
<td>3.679 +/- 0.367</td>
</tr>
<tr>
<td>F3</td>
<td>5.687 +/- 0.437</td>
<td>1.551 +/- 0.656</td>
</tr>
</tbody>
</table>

*ND stands for non-detectable, indicating that the level of *L. monocytogenes* in the sample is below the limit of detection of 1.522 log MPN/mL.
Figure B.3.3 Summary of Treatment 2 SSOP

Debris Removal, Pre-Rinse, Pressure Wash Brushes

Apply Wax Strip Plus in wax area for 15 minutes and rinse

Apply Foam Cleaner (1.4 gal of cleaner to 20 gallons of water), Scrub for 15 Minutes Wet Packingline and Rinse

Apply PAA Sanitizer (0.5 fl. oz. to 1 gallon of water)

Figure B.3.4 Summary of Treatment 3 SSOP

Debris Removal, Pre-Rinse, Pressure Wash Brushes

Apply Wax Strip Plus in wax area for 15 minutes and rinse

Apply Foam Cleaner (1.4 gal of cleaner to 20 gallons of water), Scrub for 15 Minutes Wet Packingline and Rinse

Apply acid cleaner to stainless steel areas of the line for 10 minutes and rinse

Apply PAA Sanitizer (0.5 fl. oz. to 1 gallon of water)
Figure B.3.5 Summary of Treatment 4 SSOP

1. **Debris Removal, Pre-Rinse, Pressure Wash Brushes**

2. **Apply Wax Strip Plus in wax area for 15 minutes and rinse**

3. **Apply Foam Cleaner (1.4 gal of cleaner to 20 gallons of water), Scrub for 15 Minutes Wet Packingline and Rinse**

4. **Apply 3 gallons of Sterilex Ultra Disinfectant and 3 gallons of Sterilex Ultra Disinfectant Activator into 30 gallons of water, let sit for 10 minutes, rinse, and repeat 2x**

5. **Apply PAA Sanitizer (0.5 fl. oz. to 1 gallon of water)**
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