The Pennsylvania State University

The Graduate School

BUILT ENVIRONMENT MICROBIOTA ASSOCIATED WITH OCCURRENCE OF *LISTERIA MONOCYTOGENES* IN TREE FRUIT PROCESSING FACILITIES

A Thesis in

Food Science

by

Xiaoqing Tan

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Submitted in Partial Fulfillment
of the Requirements
for the Degree of

Master of Science

August 2019

The thesis of Xiaoqing Tan was reviewed and approved* by the following:

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

Jasna Kovac
Assistant Professor of Food Science
Thesis Co-Advisor

Edward G. Dudley
Professor of Food Science

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

*Signatures are on file in the Graduate School

Abstract

Listeriosis, an infection caused by the foodborne pathogen *L. monocytogenes*, has 20 to 30% fatality rate in high-risk groups. In 2014, an outbreak linked to consumption of contaminated packaged caramel apples caused 34 hospitalization and 3 deaths, understanding methods for controlling *L. monocytogenes* in the apple supply chain became a high priority.

We conducted a longitudinal study in three tree fruit packing facilities in Pennsylvania to determine the occurrence of *L. monocytogenes* in these facilities and to investigate the association between facility environmental microbiota and the occurrence of *L. monocytogenes*. Samples were collected from zone 3 non-food contact surfaces underneath wet processing sections over a 6-month period to identify *L. monocytogenes* occurrence using the FDA BAM enrichment method, as well as to characterize the composition of the background microbiota through high-throughput sequencing.

Alpha diversity and beta diversity analysis revealed compositional differences in environmental microbiota between the facility with the highest *L. monocytogenes* occurrence level and two facilities with lower *L. monocytogenes* levels. The bacterial family Pseudomonadaceae and fungal family Dipodascaceae were found to be the predominating microorganisms facility with high occurrence of *L. monocytogenes*, suggesting potential symbiotic interactions between non-pathogenic microorganisms and *L. monocytogenes*. The predicted bacterial functional profiles of microbiota from three investigated facilities helped in further inference of putative mechanisms that may contribute to the observed compositional difference in microbiota.

To conclude, our results showed that the composition and diversity of facility environmental microbiota was associated with occurrence of *L. monocytogenes* in apple packing facilities, and that the microbiota may potentially affect the persistence of *L. monocytogenes*. These results provide a new insight into potential microbial interactions in fresh produce processing facilities that may guide further studies focused on development and testing of *L. monocytogenes* control strategies to improve fresh produce safety and quality.

TABLE OF CONTENTS

List of Abbreviations

ALOA	Agar *Listeria* Ottavani & Agosti
ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
BAM	Bacteriological Aanalytical Manual
BLEB	Buffered *Listeria* Enrichment Broth
CDC	Center for Diseases Control
CFSAN	Center for Food Safety and Applied Nutrition
CFU	Colony Forming Unit
DNA	Deoxyribonucleic Acid
EPS	Extracellular Polymeric Substance
EU	European Union
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Service
FSMA	Food Safety Modernization Act
iap	Invasive Associated Protein
ITS	Internal Transcribed Spacer
KEGG	Kyoto Encyclopedia of Genes and Genomes
LD	Lethal Dose
MPN	Most Probable Number
ORA	Office of Regulatory Affairs

OTU	Operational Taxonomic Unit
PBS	Phosphate Buffer Saline
PCoA	Principal Coordinates Analysis
PCR	Polymerase Chain Reaction
PERMANOVA	Permutational Multivariate Analysis of Variance
PICRUSt	Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PTFE	Polytetrafluoroethylene
QAC	Quaternary Ammonium Compound
RA	Relative Abundance
RPM	Revolutions Per Minute
rRNA	Ribosomal Ribonucleic Acid
RTE	Ready-To-Eat
TSAYE	Tryptic Soy Agar with 0.6% Yeast Extract
TSBYE	Tryptic Soy Agar with 0.6% Yeast Extract
USDA	United States Department of Agriculture

LIST OF FIGURES

LIST OF TABLES

Acknowledgements

I would like to express my tremendous gratitude to all people who helped me walk through the graduate school journey. I would like to thank my advisor Dr. Luke LaBorde for giving me the opportunity to pursue my master degree at Penn State University and for his guidance on this project. I would like to thank my advisor Dr. Jasna Kovac, who helped me develop professionally and had tremendous input on this project. I would like to thank my committee member Dr. Edward Dudley for insightful suggestions on my research, and for providing access to Miseq so I was able to obtain my data in a timely manner.

I would like to thank all my friend in lab, Xiaoxuan, Taejung, Manjary, Tobin, Runan, Laura for their companies in all up- and down- time. And all my friends who were or weren't physically present, Mohan, Francesca, Irene, hy and hxf, I could not finish the journey without their love and support. In particular, to my CTS group for all laughters, tears and moments we shared together in the past two years.

I would like to thank Department of Food Science and Penn State for their financial support. I would like to thank all food science faculties, staffs and graduate students for their help along the way. Last but not least, I would like to thank my mom Hongchun Li and my dad Jinsong Tan, for their love, care, and encouragement throughout my life

Chapter 1

Statement of the problem

Listeria monocytogenes is a facultative anaerobic foodborne pathogen that is nearly ubiquitously present in the natural environment. It can cause an infectious disease called listeriosis which has 20 to 30% of fatality rate in the high-risk groups including young, old, pregnant and immunocompromised people. *L. monocytogenes* is of great concern in the food industry and has been historically associated with outbreaks and recalls of a variety of food categories including ready to eat (RTE) foods, raw milk, and fresh produce.

Fresh vegetable and fruits are increasingly gaining in popularity due to their high nutrient content that is preserved when only minimally processed. However, consumption of fresh produce increases human exposure to pathogens that are transmitted from natural pre-harvest environment onto food products. Due to the lack of the thermal processing step, the foodborne pathogens transmitted to produce are likely to survive and therefore present increased risk for human exposure. In 2014, a multistate foodborne outbreak of listeriosis was reported. It was traced back to contaminated prepackaged caramel apples that caused 34 hospitalization and 7 deaths. Recently, *L. monocytogenes* contamination has been associated with sliced apples that had been recalled.

The US is the second largest apple production country, and apple consumption was approximately 44 pounds per person in 2012. Pennsylvania ranked as the 4th apple producing state with 400 to 500 million pounds of apples produced per year (USApple, 2014). The increasing demand for apple products and the growing concern related to microbiological safety of apples has therefore led to enhanced research efforts focused on improved understanding of occurrence and ways by which *L. monocytogenes* is introduced in apple processing facilities. Furthermore, there are gaps in knowledge related to understanding the factors that allow this pathogen to persist in food processing environments over prolonged periods of time. Better understanding of these factors would provide a foundation for evidence-driven optimization of pathogen control interventions in the apple supply chain.

Apple packing facilities serve as the intermediate link between orchard (pre-harvest environment) and retail distribution (late post-harvest environment. As such, they have been identified as one of the critical sources of pathogen contamination. Biotic factors, including high moisture, nutrients released from broken apple pieces and cold temperature inside facilities create an ideal environment for *Listeria* spp. to establish itself in difficult-to-clean niches and persist over time. Abiotic factors, such as improper usage of cleaning and disinfection reagents, and poor employee personal hygiene support this persistence and also promote the growth of a variety of others, commonly nonpathogenic bacteria and fungi. Currently, there is no published data characterizing microbiomes and mycobiomes of apple packing houses and the role of the built environment microbiota and mycobiota in persistence of *L. monocytogenes*. Limited

information on the composition of microbiome in the built environment hinders the understanding of these interaction. In order to establish effective interventions for pathogen control in the apple processing facilities, we aimed to elucidate the associations between facility microbiota and the occurrence of *L. monocytogenes* in the built food processing environment.

Metagenomic sequencing has transformed approaches to microbial community characterization and identification, and it has become an emerging tool for food scientist to leverage to enhance food safety. The purpose of this study was to integrate traditional microbiological detection of *L. monocytogenes* with metagenomic sequencing to investigate the associations between *L. monocytogenes* occurrence and the diversity of microbiota and mycobiota present in the built environment of three apple packing facilities located in Pennsylvania. We took this approach with the goal of improving our understanding of the role of the microbial communities in persistence of *L. monocytogenes* in the built apple packing house environment to facilitate optimization of *L. monocytogenes* control interventions in industry.

Chapter 2

Literature review

2.1 Characteristics of *Listeria* and *Listeria monocytogenes*

The genus *Listeria* comprises a group of gram-positive, rod-shape, facultative anaerobic microorganisms. It is taxonomically classified under Bacteria, Firmicutes, Bacilli, Bacillales and Listeriaceae. The genus *Listeria* is currently divided into two groups: *Listeria sensu stricto* (*L. monocytogenes, L. ivanovii, L. seeligeri, L. innocua, L. welshimeri, and L. marthii*) and *Listeria sensu lato* (*L. fleischmannii, L. grayi, L. rocourtiae, L. weihenstephanensis, L. floridensis, L. aquatica, L. cornellensis, L. riparia, L. grandensis, L. booriae, L. newyorkensis, L. costaricensis, L. thailandensis*) (Amajoud et al., 2018; Liao, Wiedmann, & Kovac, 2017; Skerman, McGowan, & Sneath, 1989). *L. monocytogenes* has the ability to cause an infection in humans called listeriosis. Listeriosis has a 20 to 30% fatality rate in high risks group such as young, old, pregnant and immunocompromised individuals (Charlier et al., 2017; De et al., 2005). *L. ivanovii* is an animal pathogen, causing disease primarily in ruminants. *Listeria* spp. have also been isolated from the intestinal microflora of asymptomatic ruminants, birds, and humans, suggesting that they also reside in these hosts as commensal bacteria (Dortet, Veiga-Chacon, & Cossart, 2009). In this thesis, we focus on the human foodborne pathogen *L. monocytogenes*.

2.1.1 *Listeria monocytogenes* – a foodborne pathogen

L. monocytogenes is ubiquitous in the natural environment including soil, surface waters, and plants (Jingjin Wang, Ray, Hammons, & Oliver, 2015). In agricultural environments, *L. monocytogenes* has been frequently detected in close proximity to pastures and areas with high moisture levels (Strawn et al., 2013). *L. monocytogenes* grows optimally at temperatures between 30 to 37 ºC (Soumet, Ragimbeau, & Maris, 2005), at pH values between 6 and 8, at salt concentrations of up to 0.5%, and in environments with water activity level above 0.924 (Wijtes, McClure, Zwietering, & Roberts, 1993). A combination of several of these extrinsic factors is commonly found in wet food processing environments, hence survival and growth of *L. monocytogenes* is of major concern in the food industry. Because of its ability to grow in biofilms, *L. monocytogenes* can adapt to disinfection agents through adaptation and development of genetic resistance to sanitizers routinely used in food processing facilities. The term resistance and tolerance are sometimes applied interchangeably, which can lead to inaccurate characterization of sensitivity toward antimicrobial treatment (Brauner, Fridman, Gefen, & Balaban, 2016) . Resistance is typically caused by genetic change such as mutation in bacterial strains, and hence higher concentration of antimicrobial is required to achieve the same effect in these resistant strains compared to wild type strains (Chait, Craney, & Kishony, 2007); on the other hand, tolerance is generally referred as the ability to survive a temporary exposure to antimicrobial treatment (Brauner et al., 2016). In addition to developing tolerance or resistance to antimicrobials, *L. monocytogenes* can survive in suboptimal conditions such as temperatures between -0.4

to 45 ºC (Farber & Peterkin, 1991), pH between 3.0 to 12.0 (D. Liu, Lawrence, Ainsworth, & Austin, 2005), and salt concentrations of up to 40% w/v, which contributes to its persistence in post-harvest food processing environments. *L. monocytogenes* can also dwell in the human intestinal tract as part of the natural microflora; between 2% and 10% of the human population carry this microorganism without showing any apparent disease symptoms (Buchanan, Gorris, Hayman, Jackson, & Whiting, 2017).

2.1.2 Pathogenesis of *L. monocytogenes* and risk assessment

L. monocytogenes is an intracellular foodborne pathogen. The invasive strains invade and grow in mammalian host cells where they can induce the infectious disease (Dabiri, Sanger, Portnoy, & Southwick, 1990; Gaillard, Berche, Mounier, Richard, & Sansonetti, 1987). Listeriosis is generally manifested as one of the following clinical syndromes: neonatal infection, blood stream infection, meningoencephalitis, and febrile gastroenteritis (Charlier et al., 2017; Donovan, 2015). Listeriosis was first recognized as a human and animal infection caused by bacteria in 1920s (McLauchlin, 1997). However, it was not until 1981 that scientists linked a foodborne outbreak to *L. monocytogenes*, which initiated investigations of mechanisms of pathogenesis and routes of transmission of this pathogen (McLauchlin, 1997*). L. monocytogenes* can cause severe disease conditions such as septicemia, encephalitis, meningitis, abortions, and stillbirths in high risk groups (Swaminathan & Gerner-Smidt, 2007). Immunocompromised individuals are especially susceptible to *L. monocytogenes* infection due toT-cell dysfunction, since cell-mediated immunity is the main body defense system against *Listeria* (Lara-Tejero &

Pamer, 2004). In addition to immunocompromised individuals, the elderly, children, and pregnant women are at increased risk for listeriosis infection after exposure to *L. monocytogenes* (Swaminathan & Gerner-Smidt, 2007). Non-invasive listeriosis has also been shown to cause symptoms such as febrile gastroenteritis, however, the pathogenic mechanisms are not well understood (Halbedel et al., 2019).

Pathogenesis of invasive *L. monocytogenes* starts with bacterial invasion of epithelial cells in the gastrointestinal (GI) tract, mediated by two surface proteins, InlA (internalin A) and InlB (internalin B). After epithelial invasion, the bacteria can enter the bloodstream via endocytosis. Listeriolysin O, a bacterial pore-forming toxin produced by hemolytic strains of *L. monocytogenes,* promotes lysis of vacuolar together with bacterial phospholipases PlcA and PlcB (Pizarro-Cerdá, Kühbacher, & Cossart, 2012). Cells are able to escape and evade cytoplasm where the bacteria start to replicate (Southwick & Purich, 1996) (Figure 2.1).

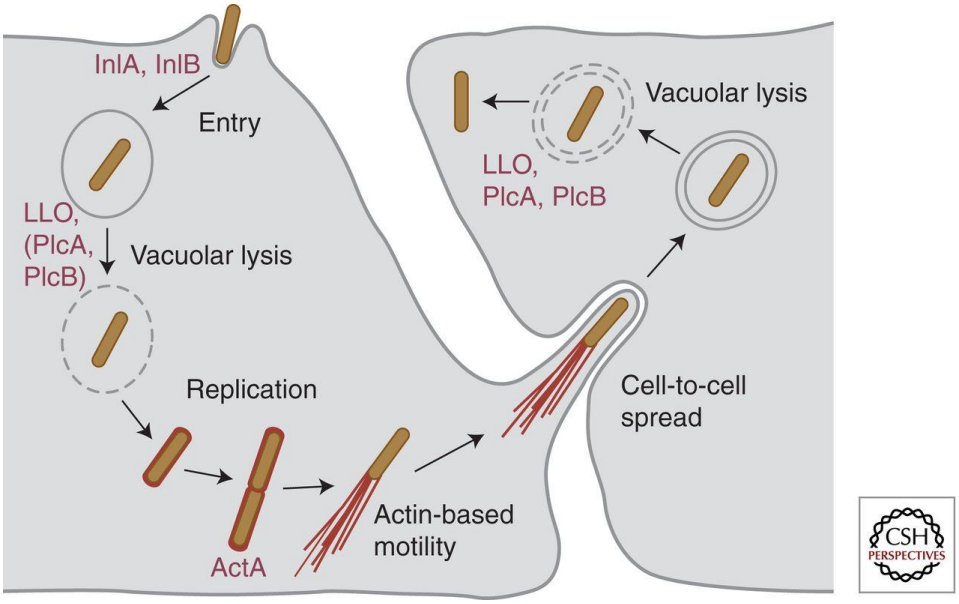

Figure 2. 1: Intracellular cycle of *L. monocytogenes* infection (Pizarro-Cerdá et al., 2012).

The process described above results in systemic infection, organ damage, or spontaneous abortion in pregnant woman (Pentecost, Otto, Theriot, & Amieva, 2006). The most notable body regions affected by the infection include the central nervous system, the placenta, and the liver (Pentecost et al., 2006). Based on epidemiological data, not all strains of *L. monocytogenes* have the same ability to cause disease in humans. Previous studies have shown that *L. monocytogenes* can be classified into four lineages; I, II, III, and IV (Orsi, Bakker, & Wiedmann, 2011; Ragon et al., 2008). Lineage I (serovars 1/2b and 4b) and lineage II (serotype 1/2a) strains are most commonly associated with human clinical cases occurring during listeriosis outbreaks, while lineage II strains are more often associated with isolates sampled from food or food processing environments. To date, serovars 1/2a, 1/2b and 4b were responsible for 98% of all human listeriosis cases (Dortet et al., 2009), with most outbreaks and sporadic cases

caused by serovar 4b (Dortet et al., 2009). Lineage III and IV strains are rare and are mainly isolated from animal sources (Orsi et al., 2011).

Typically, it takes several weeks to show symptoms of infection from exposure to *L. monocytogenes*, which makes it very difficult to acquire accurate dose information from epidemiological data (Buchanan et al., 2017). Dose-response curves have been constructed using animal models including mice, gerbils, guinea pigs and non-human primates (Notermans, Dufrenne, Teunis, & Chackraborty, 1998; Roulo, Fishburn, Amosu, Etchison, & Smith, 2014; Williams, Castleman, Lee, Mote, & Smith, 2009). Based on these models, the median lethal dose (LD_{50}) is approximately 10^7 CFU. The LD_{50} value estimated by the World Health Organization (WHO) for human listeriosis is 1.9×10^6 CFU (Nations, 2004).

According to the risk assessment in 2010 by FDA and FSIS, environmental factors that increase the risk for contamination of food products include cross contamination from food contact and non-food contact surfaces, inadequate sanitation practices, inadequate temperature control, and poor employee hygiene (Quesenberry et al., 2010). Thus, food produced in processing environments with inadequate preventive controls, is more likely to become contaminated with *L. monocytogenes* (J. M. Lundén, Miettinen, Autio, & Korkeala, 2000; Ryser & Marth, 1999; Sauders et al., 2006; Tompkin, 2002).

2.2 Food safety regulations pertaining to *L. monocytogenes*

Listeriosis outbreaks have been reported worldwide, and have been linked to food products that are exported to or imported from other countries (Jemmi, Pak, & Salman, 2002). Nevertheless, there is currently no international agreement on the regulation of *L. monocytogenes* in food, and different criteria for *L. monocytogenes* tolerable levels have been established in different regions. In the U.S., the Food and Drug Administration (FDA) established a zero-tolerance limit for *L. monocytogenes* in Ready-to-Eat (RTE) foods, which is defined as equivalent to absence in 25g (< 0.04 CFU/g), as determined in microbiological testing. In response to a petition submitted by 15 U.S. trade associations, FDA recently issued a new draft guidance which separated foods into two categories (FDA, 2008).The microbiological criteria for foods that support the growth remains at absence in 25 g (< 0.04 CFU/g); whereas foods that do not support the growth of *L. monocytogenes* have the newly established microbiological criteria of less than 100 CFU/g (FDA, 2016). The new criteria are more closely aligned with the criteria established by the EU and Canada with only minor difference in the food categories (Health Canada, 2011; Ricci et al., 2018). For instance, Parma ham, a product produced exclusively in a limited area in Italy, has faced different regulations of the presence of *L. monocytogenes* by the importing countries. European Union Member States, Canada, and Japan, have set the limit of 100 CFU/g for importing Parma ham whereas the U.S. requires absence of *L. monocytogenes* in 25 g of Parma ham sample (Morganti et al., 2016).

The Food Safety Modernization Act (FSMA), signed into law in 2011, gives FDA new legislative authority for regulation of food safety in food facilities, establishment of safe produce standards, mandatory recalls for contaminated food, and imported food regulations to better protect public health (Silk et al., 2013). The Produce Safety Rule under FSMA sets regulatory standards for harvesting, handling and production of vegetables and fruits in an effort to reduce the risk of pathogen contamination of fresh produce (CFSAN). The Preventive Controls for Human Food Rule is another regulation under FSMA that oversees the food processing and produce packing operation system to prevent pathogen contamination in the processing environment (FDA, 2018). It requires facilities to implement a food safety plan which outlines a hazard analysis, preventive control, management of preventive controls, supply chain program, and a recall plan (FDA, 2018). According to the FDA Draft guidance for Industry: Control of *Listeria monocytogenes* in Ready-To-Eat Foods, the presence of *L. monocytogenes* in food or on food-contact surfaces indicates a reasonable probability of severe health consequences or death upon ingesting the food products, and therefore is subject to class I recall in the United States (Wong, Street, Delgado, & Klontz, 2000).

The draft guidance also outlines environmental testing and monitoring procedures in processing facilities (FDA, 2017). Corrective actions may vary depending on whether *Listeria* spp. is detected on food contact or a non-food contact surfaces and the number of positive samples acquired, with the recognition that finding *Listeria* spp. is expected (FDA, 2017). Detailed corrective actions for finding *Listeria* spp. in environmental samples is summarized in Table 2.1. In short, FDA recommends

processors to conduct intensive sampling for the presence of *L. monocytogenes*, optimize sanitation procedures, conduct follow-up samplings, identify potential causes of the contamination, and document the responding corrective actions (FDA, 2017; Warriner & Namvar, 2009).

Table 2. 1: Corrective actions when *Listeria* spp. is found in an enviornmental sample. Adopted from FDA Draft guidance for Industry (FDA, 2017)

	Non-Food Contact Surfaces	**Food Contact Surfaces (support growth)**	**Food Contact Surfaces (not supports growth)**
Positive sample from Routing sampling	Clean and sanitize Retest during next production	Clean and sanitize Retest during next production Comprehensive investigation	Clean and sanitize Retest during next production Comprehensive investigation
Second positive from follow up sampling	Intensified cleaning and sanitizing Intensified sampling and testing	Intensified cleaning and sanitizing (with disassembly) Intensified sampling and testing Hold and test product Reprocess, divert or destroy product on hold if positive Comprehensive investigation	Intensified cleaning and sanitizing (with disassembly) Intensified sampling and testing Consider hold and test Comprehensive investigation
Third positive from follow up sampling	Root cause analysis	Stop production and consult food safety experts Intensified cleaning and sanitizing Intensified sampling and testing Resume production with product on hold and test until 3 consecutive days of product and FCSs are negative Consider recall	Intensified cleaning and sanitizing Intensified sampling and testing Hold and test product Expand comprehensive investigation Reprocess, divert or destroy positive product lots

2.3 *L. monocytogenes* occurrence and outbreaks

In the United States, approximately 2,500 people suffer from listeriosis annually, according to the FDA documentation, and 20% of the cases result in death (Bennion, Sorvillo, Wise, Krishna, & Mascola, 2008). Furthermore, approximately 19% of total death from foodborne illness are attributed to the consumption of *L. monocytogenes* contaminated food in the U.S. (Donovan, 2015; Scallan et al., 2011).

L. monocytogenes is mainly a food safety concern associated with ready-to-eat (RTE) foods which possess intrinsic characteristics that support the growth of vegetative cells, such as neutral pH and water activity as low as 0.93 (Farber et al., 1992; Gombas, Chen et al., 2003). Some RTE foods naturally inhibit growth of *L. monocytogenes* by their intrinsic pH and water activity characteristics, while others can be made safe by changing these characteristics through production processes such as addition of ascorbic acid (Pouillot et al., 2016). Outbreaks have been associated with food categories that are at a greater risk for contamination with *L. monocytogenes*, including raw milk (Linnan et al., 1988), soft cheese (MacDonald et al., 2005), fresh soft cheese (Centers for Disease Control and Prevention (CDC, 2001), smoked seafood (Brett et al., 1998; Ericsson et al., 1997), deli meat (Gottlieb et al., 2006; Mead et al., 2006), raw shellfish (Potasman et al., 2002). In more recent years, a number of listeriosis outbreaks have been linked with fresh produce such as packaged salads, bean sprouts and apple-associated products (Alpas et al., 2017; Garner & Kathariou, 2016; Li et al., 2017; Yeni et al., 2016).

2.4 *L. monocytogenes* occurrence in pre- and post-harvest environment

2.4.1 Occurrence of *L. monocytogenes* in fresh produce pre-harvest environments

Even though foodborne outbreaks related to tree fruit produce have not been attributed to preharvest contamination, pre-harvest environment does pose a potential risk in terms of serving as a source of produce contamination with pathogenic microorganism (Strawn et al., 2013). Both non-pathogenic and pathogenic microorganisms can be transferred onto the fresh produce in various ways, and cross contamination may occur when pathogenic organisms are transferred onto the surface of produce in the natural environment of orchard.

One study showed that 48 out of 174 surface water samples (e.g., from creek or pond) in New York State were positive for *L. monocytogenes* (Strawn et al., 2013). Notably, all positive samples were collected from natural water sources. This study also showed that irrigation water and runoff water from livestock farms is frequently contaminated with *L. monocytogenes* (Strawn et al., 2013). Another study stated that irrigation water that is contaminated with fecal matter has a high probability of containing foodborne pathogens, and has been identified as a source of produce contamination (Sadovski et al., 1978). Wilkes et al. reported that the highest prevalence of *L. monocytogenes* in water samples was in the spring (57%) (Wilkes et al., 2011). Furthermore, the prevalence of *L. monocytogenes* in the pre-harvest farm environment is significantly related to the distance from upstream cattle and dairy farms as well as the degree to which land is cropped (Lyautey et al., 2007; Sauders et al., 2006). Ruminants

can carry high levels of *L. monocytogenes* without showing any symptoms of infection. When these animals shed the pathogen in the natural environment, the risk of produce cross-contamination with *L. monocytogenes* increases (Nightingale et al., 2004). Pathogen transmission can be further accelerated by heavy rain that leads to increased bacterial levels in water (Crabill et al., 1999; O'shea & Field, 1992)

Soil can be another factor in cross contamination, since bacteria such as *Salmonella* and *L. monocytogenes* can survive for months in sewage sludge used for agricultural soil amendment (Watkins & Sleath, 1981). Choice of fertilizer could introduce different microbiota (in this context, microbiota or microbial communities include both bacterial and fungal microorganisms) onto produce and may modify the existing microbiome on the surface of the produce (Doty, 2017). Unhygienic practices during harvest, improperly treated sewage, and poorly sanitized transportation trucks also represent potential factors for foodborne pathogen transmission to post-harvest produce processing environment (Robert & Brackett, 1999). *L. monocytogenes* is known for its ability to adapt, persist in human-associated indoor habitats (built environment) (Adams et al., 2015), where it can contaminate food products over time. Here we specifically focus on the occurrence of *L. monocytogenes* and associated microbiota and mycobiota in indoor food processing environments of apple packing houses that provide ample moisture and nutrient supply that are known to support *L. monocytogenes* growth and persistence (Thimothe et al., 2004).

2.4.2 Occurrence of *L. monocytogenes* in packing and processing environments

The food processing environment has been identified as an important source of pathogens that contaminate food (Beuchat, 1996; Chasseignaux et al., 2001). *Listeria* can enter food processing environments and disperse through various ways including raw material receiving and transportation between wet and dry processing areas (Fox et al., 2011). When the food processing environment becomes contaminated, the risk for the pathogen transmission to food and for the human exposure increases (Tompkin, 2002). Hence, it is critical to have effective pathogen control strategies in place to prevent adaptation and persistence of *L. monocytogenes* in the food processing environment over time.

The term "persistence" is used in this context to describe long-term survival and therefore frequent detection of the same clone of a pathogen, in this case *L. monocytogenes*, over time in complex natural or built-environments (Ferreira et al., 2014; Lundén et al., 2003). Many factors can cause persistence of *L. monocytogenes*, such as poor equipment design, continuous moisture and food debris, and insufficiently trained employees. Difficult-to-clean areas such as conveyor belt rollers, metal-to-metal connections, cracked rubber seals, insulation and switches have all been identified as sources of persistent *Listeria* in commercial RTE meat and poultry production facilities (Malley et al., 2015; Tompkin, 2002). Persistent *Listeria* have also been found in a fresh mushroom packing and slicing environments, frozen vegetable processing environments and onion slicing equipment (Aguado, 2004; Murugesan et al., 2015; Scollon et al., 2016). Persistence of *L. monocytogenes* may be attributed to their ability to form biofilms

that attach onto the surfaces (Skowron et al., 2019). *L. monocytogenes* was shown to be able to attach, colonize and produce biofilms on different surfaces that are commonly used in the food industry, including polystyrene, glass, rubber, and stainless steel (Mikš-Krajnik et al., 2016; Ripolles-Avila et al., 2018). The following section will be focused on how *L. monocytogenes* is controlled in the post-harvest packing and processing facilities.

2.4.2.1 Control of *L. monocytogenes* in post-harvest food processing environment

In food processing facilities, especially where RTE foods are produced, environmental cleanliness is crucial to food safety, and is heavily reliant on the appropriate use of cleaning and sanitizing agents. Common antimicrobial compounds used to eliminate pathogenic microorganisms on foods or in food processing environments include bacteriocins, organic acids, antibiotics, and sanitizers. Here we focus on sanitizers that are most commonly used in the post-harvest produce processing environment.

Commonly used sanitizers in the food processing environment including benzalkonium chloride, hydrogen peroxide, peracetic acid, and sodium hypochlorite (Block, 2001). Benzalkonium chloride is an example of a quaternary ammonium compound (QAC), a class of cationic sanitizers widely used as surface disinfectant. QACs antibacterial activity causes disintegration of the bacterial cytoplasmic membrane and leakage of intracellular material, and the residual germicidal effect has been found to be very effective against *L. monocytogenes* (Galié et al., 2018). However, some other

studies have shown that the efficacy of QACs can be reduced in the presence of organic material and biofilms, and that subinhibitory concentrations may cause sanitizer tolerance of *L. monocytogenes* (Martínez-Suárez et al., 2016; Mosteller & Bishop, 1993).

Hydrogen peroxide is commonly used as a disinfectant or as an ingredient in sanitizer mixtures. It has a powerful oxidizing effect toward bacteria and kills bacteria by generating free radicals that cause bacterial DNA damage (Clapp et al., 1994). It is considered environmentally friendly because it degrades to harmless oxygen and water (Guo et al., 2017). Hydrogen peroxide, like some other sanitizers used in aqueous solution, is unstable and easily decomposes to its waste products (Ölmez & Kretzschmar, 2009).

Peracetic acid is formed by reaction of hydrogen peroxide and acetic acid, and has antibacterial activity at lower concentration than hydrogen peroxide. It is commonly used for antimicrobial treatment of food contact surfaces (Korany et al., 2018). Peracetic acid is also environmentally friendly as it decomposes to non-toxic acetic acid and oxygen (Ceragioli et al., 2010). Peracetic acid is advantageous because it is resistant to peroxidase inactivation. Moreover, peracetic acid does not react with proteins and no production of toxic compound has been found (Rossoni & Gaylarde, 2000). The disadvantage of using this compound is that it is unstable at higher concentration (15%) and it is relatively expensive compare to other commonly used sanitizers (Vandekinderen et al., 2009).

Sodium hypochlorite is another sanitizer used extensively in the food processing environment (Fukuzaki, 2006). It dissociates into Na^+ and hypochlorite ions (OCl^-) in

water, which generates superoxide anions and hydroxyl radicals that damage bacterial cells (Dukan & Touati, 1996; Fukuzaki, 2006). Due to its strong oxidation reaction, it is important to limit the concentration to a safe level since it might be corrosive to certain metals. Moreover, the presence of organic matters may reduce the efficacy of sodium hypochlorite (Sun et al., 2012).

2.4.2.2 Biofilm formation and development of *L. monocytogenes* tolerance to sanitizers

Many studies have reviewed the impact that biofilms in the food processing environment have on increasing food safety risks to the public (Álvarez-Ordóñez & Briandet, 2016; Bridier et al., 2015; Overney et al., 2017). A biofilm is usually described as a collective population of bacteria within an extracellular matrix that are irreversibly attached to a surface (Colagiorgi et al., 2017). It can be characterized as a collection of attached cells that are cross-linked by extra-polysaccharide or extracellular polymeric substances (EPSs) produced by some members of the biofilm. EPSs are natural polymers that encase the cells within the biofilm. Other substances that make up a biofilm are proteins, nucleic acids, polysaccharide, lipids, dead cells, and other polymerics that are hydrated to 85 to 95% water (da Silva & De Martinis, 2013; Flemming et al., 2016; Sutherland, 1982). The EPS mediates adhesion of bacteria to the abiotic surface, and also protect cells from cleaning and sanitizing solutions by forming a multi-dimensional polymeric network (Singh et al., 2017). EPS also functions as an external digestive system where extracellular enzymes degrade and metabolize biopolymers into lower

molecular masses that can be utilized as nutrients and energy sources by cells in a biofilm (Flemming & Wingender, 2010).

There are five key steps in biofilm formation as illustrated in Figure 2. The first step is the reversible attachment of planktonic cells to the surface. The attached cells can be easily removed at this stage. In the cell attachment step, the reversibly adhered cells no longer detach, and at this stage the cell adherence becomes irreversible. The community of three to five layers of bacterial cells are referred to as microcolonies. In this stage, cells start to aggregate and produce EPS which is a predominant structure that facilitate stable cell to cell interactions within a biofilm (Stanley & Lazazzera, 2004). The true biofilm structure is fully developed in stage three, when cells cluster and form a multi-layer matrix. Biofilms are thick formations with complex structures consisting of channels that allow for nutrient influx and waste efflux. The maturation of the biofilm is achieved by a combination of nutrient and quorum sensing signals which quantity increases with increased cell density (Miller & Bassler, 2001). Finally, in the cell detachment stage, cells aggregate and form a three-dimensional structure that helps them become resistant to cleaning and disinfection. Once cells in the biofilm reach stage four and begin to saturate, they gradually disperse back into the surrounding environment as planktonic cells where they can restart the biofilm formation cycle from the stage one at another location (Ha & O'Toole, 2015; Rasamiravaka et al., 2015; Stanley & Lazazzera, 2004).

The outer surface structure of individual cells determines the biofilm properties and the extent to which biofilm attachment occurs (Strevett & Chen, 2003). Flagella, possessed by many bacteria for the purpose of motility play a crucial role in establishing

contact with abiotic surfaces (Acemel et al., 2018; Kostakioti et al., 2013). The mutant of *Pseudomonas fluorescens,* which lacks flagella, has been shown to have a reduced ability to attach to abiotic surfaces compared to the highly motile wild type, thus confirming the important role of flagellar motion in the initial stage of the biofilm formation (Amores et al., 2017; Armbruster & Parsek, 2018). Other surface structures like pili (fimbriae) and curli may also support active attachment of bacteria to surfaces and contribute to the cell adhesion, which is a first step of the biofilm formation (Figure 2.2) (Cookson, Cooley, & Woodward, 2002; Crouzet et al., 2014; Mandlik et al., 2008)

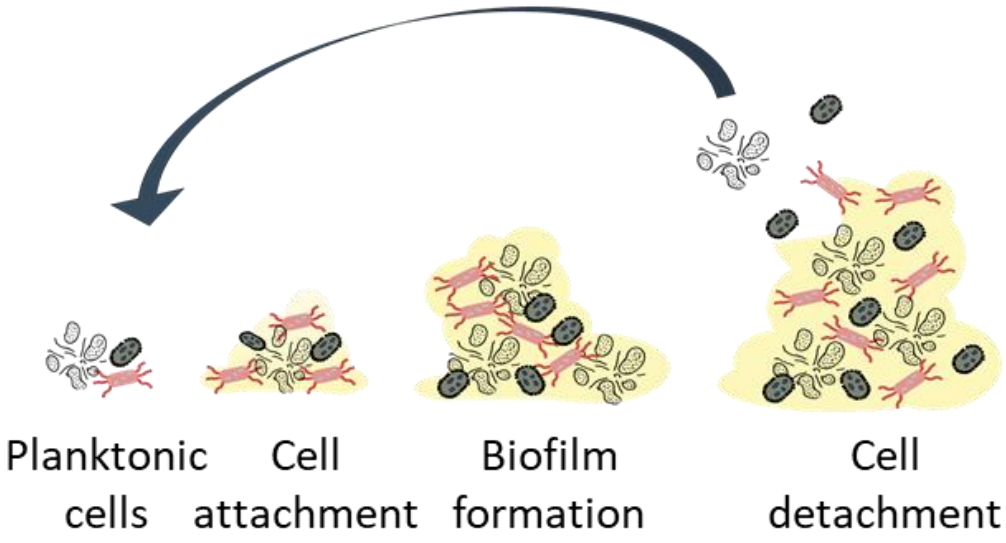

Figure 2. 2: Process of biofilm formation.

With respect to *L. monocytogenes*, it can exist in planktonic form or attach to surfaces and form a biofilm. *L. monocytogenes*, by itself, can only produce a thin biofilm, and polysaccharides are rarely detected in *L. monocytogenes* biofilm unlike other strong biofilm formers such as *Pseudomonas* spp. (Doijad et al., 2015; Guilbaud et al., 2015). However, multispecies biofilms containing *L. monocytogenes* in the food processing

environment are prevalent (Puga et al., 2018). The ability of *L. monocytogenes* to form biofilm is dependent on strain, medium, types of surface, and environmental conditions (Kadam et al., 2013). *L. monocytogenes* is able to attach on polytetrafluoroethylene (PTFE) surfaces, one of the most hydrophobic materials, which is often used as "non-stick" coating and sometimes used as s material for tanks, pipping and their connections in the fresh produce industry (Fysun et al., 2019; Rosenblum et al., 2012).

The ability of *L. monocytogenes* to form a biofilm was also shown to be lineage-dependent (Valderrama, Ostiguy, & Cutter, 2014). Lineage II strains have been shown to have higher ability to form biofilms on stainless steel compared to tested lineage I stains when grown in nutrient-limited conditions (Djordjevic et al., 2002). However, lineage I strains showed a denser biofilm formation under nutrient-rich conditions (Takahashi et al., 2010). This demonstrates that the growth medium can have a great impact on the ability of cells to form biofilms and that experimental study designs should take in consideration the conditions of the environment that they aim to mimic. It is also shown that the ability for *L. monocytogenes* to form biofilm increases when the incubation temperature increased from 20 ºC to 30 ºC (Nowak et al., 2017).

Persistent strains have shown to produce thicker biofilms compared to transient strains in the food environment (Colagiorgi et al., 2016), and Nakamura et al. also showed that EPS production in biofilms is greater in persistent strains of *L. monocytogenes* compared to transient strains, which may play a role in the greater sanitizer tolerance of the persistent *L. monocytogenes* strains in the processing environment (Nakamura et al., 2013). Another study carried out by Chaitiemwong et al.

demonstrated that the presence of food residues and biofilm can greatly reduce the effectiveness of disinfectants on surfaces with grooves (Chaitiemwong et al., 2014). The presence of biofilms could be a challenge for effective cleaning and sanitizing in the food production environment, and thus may serve as a source of contamination of pathogenic microorganisms, such as *L. monocytogenes.*

2.4.2.3 Exposure of *L. monocytogenes* to subinhibitory concentrations of sanitizers and subsequent adaptation to sanitizing agents

L. monocytogenes can develop tolerance or resistance to sanitizers used in the food processing environments, leading to inadequate control of the pathogen (Dutta et al., 2013; Ortiz et al., 2014; Rodríguez-Melcón et al., 2018). *L. monocytogenes* often develops tolerance after repeated exposure to sanitizers, especially if applied in subinhibitory concentrations. This was demonstrated in a study of Pan *et al*, where a mixture of peroxyacetic acid, hydrogen peroxide, and octanoic acid sanitizers could effectively reduce *L. monocytogenes* levels during the first week of the experiment, however, cells developed tolerance after forming biofilms on coupon surfaces (Pan et al., 2006). Another study compared the effect of sanitizers against planktonic and in-biofilm *L. monocytogenes* cells. The results of testing acetic acid, NaOH, 10% Na_2SO_4 and acetic acid, 10% Na_2SO_4 and NaOH, quaternary ammonium, and glyceryl monolaurate demonstrated that alkaline sanitizers were effective against both planktonic cells and attached cells. However, tolerance was observed after 7 days after initial adhesion of biofilm (Chavant et al., 2004). Other studies reported similar findings indicating

development of *L. monocytogenes* tolerance toward sanitizing agents when in attached form (Oxaran et al., 2018; Rodríguez-Melcón et al., 2018)

Other studies compared the effectiveness of commercially available sanitizers against *L. monocytogenes*. Yang et al. found 9 out of 10 sanitizers (all except the quaternary ammonium compound-based sanitizer) reduced levels of five human disease-associated *L. monocytogenes* strains from 6 log CFU/cm^2 to less than 0.60 log CFU/cm^2 at day 0. However, when they repeated the same treatments on 7 and 10 day-old-*Listeria*-biofilms, the efficacy of sanitizers decreased significantly ($p < 0.05$) (H. Yang et al., 2009). These sanitizers tend to lose their efficacy on cells after cells become enclosed in a biofilm. Most studies agree that even though sanitizers like QAC are effective in inhibiting the growth of *L. monocytogenes*, tolerance of cells toward sanitizing agents increases as the cells progress from planktonic form to a biofilm (Chavant et al., 2004; Luque-Sastre, Fox, Jordan, & Fanning, 2018). Biofilm formation therefore supports the persistence of *L. monocytogenes* in the food processing facility environment.

2.4.2.4 Equipment with unsanitary design that is difficult to clean may present a source of *Listeria monocytogenes* contamination

L. monocytogenes has been frequently detected in the food processing facilities that have equipment with complex, unsanitary design. Studies have shown that, even though raw material may be the original source of *L. monocytogenes*, the strains isolated from final products are usually not the same as the strains in the raw materials (Thévenot, Dernburg, & Vernozy-Rozand, 2006). Moreover, *L. monocytogenes* strains that are

responsible for listeriosis outbreaks can usually be traced back to processing environment and equipment, suggesting that the equipment can serve as a niche for enrichment of *L. monocytogenes* (Martínez-Suárez et al., 2016; Nakari et al., 2014). Another study conducted by Berrang et al. detected *L. monocytogenes* in floor drains before and after the sanitation process, suggesting inadequate cleaning and sanitizing efficacy, possibly due to difficult-to-clean elements within the drain (Berrang et al., 2010). Other studies also identified that floor and drains have a high risk of *L. monocytogenes* persistence in the processing environment due to the unease of cleaning (Gudmundsdóttir et al., 2005; Kastbjerg & Gram, 2009; Simmons & Wiedmann, 2018). Insufficient cleaning and sanitizing allow *L. monocytogenes* as well as the other native microbiota persistence. Currently, we do not have a comprehensive understanding of the processing environment microbiota and their role in promoting or suppressing *L. monocytogenes* growth in the processing environment.

2.5 Occurrence of *L. monocytogenes* in the apple supply chain

2.5.1 Overview of the apple industry

Apples are among the most popular fruits consumed worldwide, with 84 million tons produced in 2014 (Musacchi & Serra, 2018). The U.S. contributes 6.1% to the world apple production which ranks it as the second largest apple producing countries in the world, following China. Top apple production states ranked in order are Washington, New York, Michigan, and Pennsylvania. There are 7,500 apple growers managing 324, 000 acres of apple orchards in the U.S., and the average consumption per person was

about 44 pounds in 2012. This market size necessitates improved product quality and safety that needs to be ensured throughout the apple supply chain (FAOSTAT, 2014).

2.5.2 *L. monocytogenes* outbreaks related to apple products

In 2014, a multistate outbreak of listeriosis that caused 34 hospitalization and 7 deaths was attributed to consumption of prepackaged caramel apples (CDC, 2018). Whole genome sequencing data revealed that clinical isolates from hospitalized patients were closely related to isolates found in the facility where apples were packed (CDC, 2018). In 2017, another outbreak associated with caramel apples occurred, although the clinical strain of *L. monocytogenes* was not isolated from caramel apples nor the processing environment. Nevertheless, epidemiologic data showed that the three reported cases of listeriosis were likely consumed prepackaged caramel apples produced in the same facility (Marus, 2019).

Several recalls of sliced fresh apples have been issued due to positive *L. monocytogenes* test results of finished products. In 2014, after random sampling conducted by the Division of Food Safety of the Ohio Department of Agriculture, Del Monte Fresh Inc. recalled Pennsylvania-grown fresh-cut sliced apple products due to increased risk of fruit being contaminated with *L. monocytogenes*, although no illnesses have been reported (Salamone, 2014). In 2015, Sun Rich Fresh Foods Inc. in Canada recalled sliced apple products due to possible *L. monocytogenes* contamination. One case of illness was found to be associated with the consumption of recalled products (Government of Canada, 2015). In 2016, Fresh from Texas recalled multiple products

containing sliced apples due to detection of *L. monocytogenes* during two random samplings of the same batch of product (Office of Regulatory Affairs, 2016).

In 2014, a recall of stone fruits including whole peaches, nectarines, plums and pluots was issued (Jackson et al., 2015). After the recall, many concerned consumers made inquiries about listeriosis and also reported that they have purchased recalled products. However, only one case of listeriosis has been found to be associated with the recalled fruit based on epidemiological data (Jackson et al., 2015). Outbreaks and recalls related to apple products and tree fruits call for an urgent need for the improvement of control interventions in the apple industry, however, limited knowledge on the key control points that need to be prioritized has been provided in the past published research.

2.5.3 Pre-harvest apple orchard

A variety of microorganisms can colonize the surface of apple leaves, flowers, stems, and apples, and enter their tissues in the pre-harvest environment (Abdelfattah et al., 2016). There are different routes for these microorganisms to transfer onto plants such as from soil (Valero et al., 2007) or insects that contact the fruit (Stefanini et al., 2012). Irrigation water, air, untreated manure fertilizer, contaminated water from upstream livestock farms, and wild and domestic animals are other possible sources of produce contamination (Beuchat, 1996), and could be source of apple contamination (Figure 2.3). However, no study to date has identified the source of contamination in apple orchards.

2.5.4 Post-harvest apple packing environment

2.5.4.1 Apple packing process

Apples are harvested and transported from orchard to packing facilities by trucks, where they are transferred into a tank containing chlorinated water for cleaning and initial bacterial load reduction. Then, apples are transported on conveyor belts with rolling brushes through a wet processing section which includes washing, fan drying, and waxing. Food-grade fruit coating wax is usually added onto the surface of apples, by subjecting them to hot-air at 54 ºC for 3 to 5 seconds until the liquid wax fully dried. The waxed apples are transported on the rolling brush conveyor to the dry, packaging section of the apple packing house. Workers check the quality of finished whole apples and discard apples that are not visually suitable for selling. Undersized or damaged apples may be used for livestock feed or apple food products such as apple sauce or apple cider (Kader, 2002). After the sorting is completed, apples are either packed into boxes, bags or bins, and then immediately shipped for retail distribution, or they are stored in cold storage rooms to maintain quality and freshness.

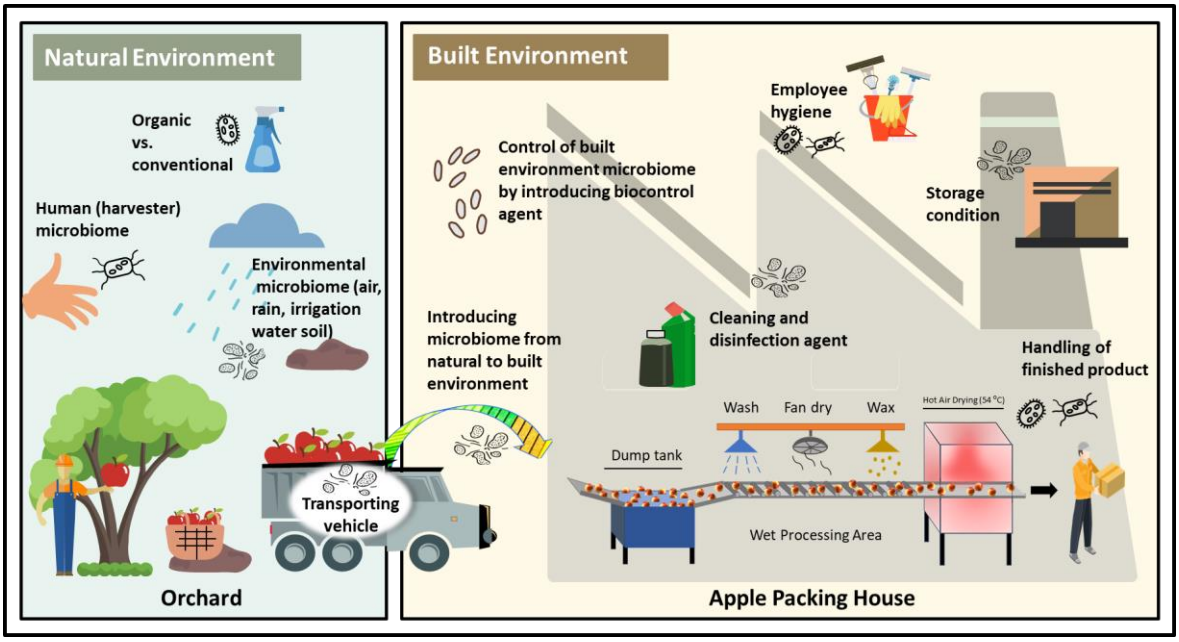

Figure 2. 3: Illustration of the microbial transmission paths and factors affecting the microbiota composition in the natural and built environment of the apple supply chain.

2.5.4.2 Built post-harvest food processing environment

The built apple processing facility environment is an intermediate link between the orchard and the retail distribution chain, and may serve as reservoir for the *L. monocytogenes* introduced onto the food product. Post-harvest food processing facilities have been identified as one of the main potential sources of food contamination with *L. monocytogenes* (Kastbjerg & Gram, 2009). Many factors, including the building and equipment design, as well as cleaning and sanitizing practices established in the facilities, affect the potential adaptation and persistence of *L. monocytogenes* in post-harvest food processing environments, including apple packing houses.

2.5.4.3 Factors influencing the survival and growth of *L. monocytogenes* in apple packing houses

Factors affecting the survival and growth of *L. monocytogenes* in apple packing houses include both biotic and abiotic factors (Figure 2.3). Apple packing facilities usually operate at lower temperature (12 ºC – 17ºC) to maintain the quality of apples. Psychrotrophic organisms like *L. monocytogenes* can survive and grow in such an environment. Moreover, the combination of high moisture, and nutrients released from damaged apples create a suitable environment for *Listeria* spp. survival and growth. Equipment such as roller brushes that transport apples through the wet processing section are challenging to clean. One of the most common areas of pathogen persistence are also floor drains that can become pathogen reservoirs because of the poor design and improper cleaning (Camargo et al., 2017). The improper use of cleaning and sanitizing agents may promote the growth of *L. monocytogenes*, as well as a variety of other microorganisms, including bacteria and fungi found in such environments.

2.5.5 Studies of *L. monocytogenes* survival and growth on apple products

A study conducted by Sheng et al. investigated the impact of storage temperatures on the survival of *L. monocytogenes* on apples (Sheng et al., 2017).The results showed that even though *L. monocytogenes* did not grow on the surface of cold stored whole apples, the number of *L. monocytogenes* cells remained unchanged for up to 12 weeks, indicating its ability to survive on apples over long periods of time (Sheng et al., 2017). *L. monocytogenes* were found to grow on apples significantly when apples were

punctured with a stick and then coated with caramel. The authors concluded that the release of the apple juice into the microenvironment and supply of sugar from caramel create a microenvironment suitable for slow growth of *L. monocytogenes* at the interface of the apple and caramel (Glass et al., 2015). This finding was supported by another study that showed that at low inoculation levels *L. monocytogenes* did not grow on fresh apples without caramel coating whereas it showed significant growth under the same conditions when inoculated on apples coated with caramel (Salazar et al., 2016). This suggested the need for *L. monocytogenes* prevention prior to packaging and distribution to reduce the pathogen contamination and associated food safety risk.

L. monocytogenes does not only survive on the apples, but also interacts with other microorganisms in the same environment. A study conducted by Conway et al. focused on studying the interactions between *L. monocytogenes* and common postharvest apple pathogens on sliced apples (Conway et al., 2000). They showed that the presence of fungi *Glomerella cingulate* enhanced the growth of *L. monocytogenes* by increasing the pH of the decayed apple tissue, whereas *Penicillium expansum* decreased the pH of decayed tissue and reduced the level of *L. monocytogenes* (Conway et al., 2000). Apple packing facilities have been investigated for routine sanitizer efficacy using the presence of spoilage fungi *Penicillium expansum* as a microbial indicator (Okull et al., 2006). Limited research has been done to provide a comprehensive understanding of microbiota in fresh produce processing facility (Gu et al., 2019), and no investigation of microbiota composition has been conducted in apple packing environment.

2.6 Microbiota interspecies interactions

Microbiota refers to the collection of bacterial communities and mycobiota to fungal communities. Microbial communities contain diverse microbial species that interact with each other and contribute to the complexity of a given ecosystem. In this section, we will focus on the interspecies interactions that may positively or negatively impact the survival and growth of *L. monocytogenes* in a microbial community.

2.6.1 Bacterial-fungal interactions

Similar to bacteria, fungi can survive in a wide range of habitats, including fresh water, soil, decaying manner, and living animals and plants (Webster & Weber, 2007). Based on environmental surveys, it is estimated that there are approximately 3.8 million species of fungi (R. Henrik Nilsson et al., 2019). Bacteria and fungi have been historically studied separately, however, increasing evidence of bacterial-fungal interactions in agricultural, clinical, and environmental settings presents a compelling rationale for taking these interactions in consideration (Frey-Klett et al., 2011; Frey-Klett et al., 2007; Kobayashi & Crouch, 2009; Moss, 2008; Wargo & Hogan, 2006) . Interactions between bacteria and fungi can namely produce different functional profiles compared to those they produce independently (Deveau et al., 2018; Tarkka et al., 2009).

Bacteria and fungi can develop and form together in biofilms, and the fungi-bacteria biofilm structure is considered as an intimate association between these two groups. Fungi may provide support for bacteria during biofilm formation by acting as a biotic surface, where they can use the hyphae as a “highway” to enhance their mobility

(Seneviratne et al., 2007). Synergistic interaction was demonstrated by Zupancic et al., where they showed that multispecies bacterial biofilm can lead to better establishment of opportunistic pathogenic fungi on rubber seals of household dishwashers (Zupančič et al., 2018). The interaction between fungi and bacteria thus promote growth and colonization to abiotic surfaces.

Bacterial-fungal interactions are not always symbiotic. In fact, in most cases, fungi and bacteria inhibit each other's growth by competing for resources (Boddy, 2016). For example, a plant-protective *Pseudomonas* species produces 2,4-diacetylphloroglucinol (DAPG), which is a very effective antifungal substance (Rezzonico et al., 2007). Some *Fusarium* species strains produce fusaric acid that inhibits the production of antifungal metabolites, including DAPG, by bacteria (Notz et al., 2002). Moreover, a study has shown that *P. aeruginosa* can inhibit the survival of *Candida albicans* by attaching onto the surface of *C. albicans* and the production of phospholipase C and phenazines (Olsson et al., 2017, p. 39). Although published literature indicates the high importance of bacterial-fungal interactions, many aspects of it are still not well understood.

2.6.1.1 Bacterial-fungal interactions in food processing environments

Previous studies have investigated fungal communities in the food processing environments. Some identified filamentous fungi in the whole meal bread processing environments that may increase the risk of spoilage and decrease the shelf life and quality of the products (Nakhchian et al., 2014; Santos et al., 2016). These studies suggested the

possibility of environmental mycobiome to be transferred onto food products. Other studies have focused on the impact of bacterial-fungal interaction on inhibition of foodborne pathogens in fermented food products such as cheese (Belessi et al., 2008; Vattem et al., 2004). The fresh produce processing environment fungal composition has also been studied. Fungi such as *Botrytis cinerea* and *Penicillium expansum* may cause spoilage of fruits and vegetables, and the mycotoxins produced by various groups of fungi including patulin and ochratoxin A may lead to health concern (Moss, 2008). Other studies also investigated the effect of fungal composition in fresh produce industry mainly to better understand the shelf life of food products (Brackett, 1992; Lin & Zhao, 2007). The composition of bacteria and fungi from fresh produce was characterized by Harding et al. (Harding et al., 2016), however, only limited studies have investigated interactions between bacteria and fungi on fresh produce (Conway et al., 2000).

2.6.2 Interactions between *L. monocytogenes* and the native microbiota in food processing facilities

Previous studies have demonstrated interactions between *L. monocytogenes* and other microflora that have both positive or negative effects on colonization of *L. monocytogenes* (Carpentier & Chassaing, 2004; Hassan et al., 2004; Leriche & Carpentier, 2000). For example, planktonic *L. monocytogenes* was shown to be able to migrate to pre-established *P. fluorescens* biofilm on coupon surface, and the attached population of *L. monocytogenes* was 1 to 2 log higher than when grew on non-*P. fluorescence* treated stainless steel coupon (Puga et al., 2018).

On the other hand, *Staphylococcus sciuri* was shown to decrease *L. monocytogenes* biofilm formation on stainless steel surfaces (Giaouris et al., 2015) his may be due to competition of these microbial species for available nutrients and/or production of extracellular substances such as negatively charged teichoic acids by *S. sciuri*, which may prevent the adhesion to the other negatively-charged substrates (Leriche & Carpentier, 2000). Furthermore, Carpentier and Chassaing demonstrated that 13% of the 29 strains of Gram positive bacteria isolated from processing environment promoted growth of *L. monocytogenes* in the biofilm when co-cultured, whereas 53% of the strains suppressed growth of *L. monocytogenes* (Carpentier & Chassaing, 2004). Another study also suggested that competitiveness of *L. monocytogenes* can vary under multi-bacterial growth conditions (Heir et al., 2018). This indicates that shifts in the composition of microbiota in the food processing environment may contribute to *L. monocytogenes* persistence in these environments.

2.6.3 Leveraging microbial interactions to develop biocontrol agents against foodborne pathogens

Biological control agents, or biocontrol agents are the microbial antagonists used to control potential pathogens in food products or post-harvest environments (Droby et al., 2016). Lactic acid bacteria (LAB) have been investigated extensively as potential biocontrol agents (Gálvez et al., 2008; Izquierdo et al., 2009; L. Liu et al., 2008; Zhao et al., 2004). Studies investigating LAB as protective (also called biocontrol) cultures have shown their efficacy when applied directly on food products to control *L. monocytogenes*

in soft and hard cheeses, sausages, cold-smoked salmon and raw chicken meat (Gálvez et al., 2008; Izquierdo et al., 2009; L. Liu et al., 2008; Maragkoudakis et al., 2009; Tomé, Gibbs, & Teixeira, 2008). LAB are also used as a bio-preservation agent because of their ability to produce lactic acid that effectively decreases the pH of foods (Stiles, 1994). Organic acids such as lactic acid, acetic acid produced by LAB have a strong antibacterial activity against other bacteria (Ponce et al., 2008). Some LAB also produce bacteriocins with anti-*Listeria* activity (L. Liu et al., 2008; Pol & Smid, 1999). For instance, select strains of *Enterococcus faecium* produce a class lla bacteriocin enterocin A, which is considered as one of the most potent known anti-*Listeria*l bacteriocins (L. Liu et al., 2008). Nisin is another antimicrobial protein produced by LAB *Lactococcus lactis* ssp. *lactis*, which is bactericidal against a wide range of Gram-positive bacteria including *Listeria monocytogenes* (Pol & Smid, 1999).

In addition to application of biocontrol strains directly on food products, LAB may also be used for pathogen biocontrol in processing environments. A study conducted by Zhao et al. showed that strains *Lactococcus lactis subsp. lactis C-1-92* and *Enterococcus durans* 152 inhibited the growth of *L. monocytogenes in vitro* and in biofilms (Zhao et al., 2004). Further, these biocontrol strains were used for control of *L. monocytogenes* in the floor drains of a poultry processing plant. Application of these two cultures was shown to significantly reduce the load of *Listeria* spp. in the floor drains (Zhao et al., 2006).

Given that chemical sanitizers frequently fail to control *L. monocytogenes* in food processing environments, the idea of leveraging natural interactions among

microorganisms is emerging as an attractive alternative pathogen control strategy (Gálvez et al., 2010). Biocontrol is a feasible and a promising complementary intervention to enhance chemical sanitizing for pathogen control, however, it requires a knowledge of native microflora and its interactions with *L. monocytogenes*. Limited information is currently available for characterization of native microbiota in fresh produce processing facilities (Gu et al., 2019). Hence, a better understanding of the core microbiota in packing and processing facilities is needed for future development of biocontrol methods with broad applications.

2.7 High-throughput sequencing for microbiota characterization

Metagenomic sequencing has rapidly advanced our understanding of microbiota composition, diversity, and function in the natural environment (Jovel et al., 2016). Whole genome sequencing or shotgun genomic sequencing allows researchers to obtain the genome sequence of the whole bacterial genome, identify functional genes, and predict virulence factors (Duncan & Patel, 2017). On the other hand, most high-throughput metagenomic technologies are based on marker genes (e.g. 16S rRNA, ITS), or amplicon sequencing to target specific phylogenetically-informative or functional genes for fast and low-cost characterization of the microbiota (Benbow et al., 2017).

2.7.1 16S rRNA sequencing for characterization of bacterial communities

16S ribosomal RNA, or 16S rRNA, is a conserved region in bacterial cells responsible for translation (Větrovský & Baldrian, 2013). There are three main

characteristics of 16S rRNA gene sequence that make it a suitable tool for taxonomic identification of bacterial species and phylogenetic analyses of microbiota. First, it is present in all known bacteria (Janda & Abbott, 2007); second, horizontal gene transfer has relatively low impact on evolution of this region (Daubin et al., 2003), and third, it is comprised of both highly conserved regions and sufficiently variable regions that are suitable for taxonomic classification (Větrovský & Baldrian, 2013). Moreover, with the >90% taxonomic identification rate at a genus level (Janda & Abbott, 2007) and 65%-83% taxonomic identification rate at species level, it is one of the most commonly used genetic markers for bacterial taxonomy identification (Mignard & Flandrois, 2006; Woo et al., 2008).

16S rRNA is approximately 1500 bp long, and has nine hypervariable regions which we refer to as V1-V9, spanning over the total length of the gene (Chakravorty et al., 2007). It is possible to compare the entire 16S rRNA region to improve bacterial taxonomy identification, however, short read sequencing technologies, such as Illumina cannot be used for this purpose (Mitreva, 2017). Due to the recognition of error caused through PCR amplification and sequencing, operational taxonomic units (OTUs) are assigned to classify groups of bacteria that are closely related to each other (Glassman & Martiny, 2018). OTUs are usually defined based on a currently established taxonomic thresholds that are equal or greater than 97% and 99% similarity in the 16S rRNA sequence to classify a bacterium into a genus and species, respectively, even though the biological and taxonomic meaning of these cutoffs is still debatable (Reller et al., 2007).

2.7.1.2 Regions of 16S rRNA used for taxonomic classification

The V1 to V9 hypervariable regions of 16S rRNA span from base position 69-99, 137-242, 433-497, 576-682, 822-879, 986-1043, 1117-1173, 1243-1294 and 1435-1465, respectively, based on the *E. coli* nomenclature system (Brosius et al., 1978; Chakravorty et al., 2007). In a study conducted by Youssef et al. on comparison between numbers of OTUs generated by nearly complete fragment and OTUs obtained from each shorter region fragments, authors concluded that fragments obtained from V1+V2 and V6 regions overestimated the OTU abundance at all taxonomy levels; whereas fragments obtained from V3, V7 and V7+V8 regions underestimated the number of OTUs at all taxonomy cutoffs. On the other side, fragments containing V4, V5+V6 and V6+V7 sequences generated OTU numbers that were equivalent to the numbers calculated using complete fragments (Youssef et al., 2009). Other studies also suggested that the V4 region is considered to be one of the most accurate for microbial classification based on amplicon sequencing (Mizrahi-Man et al., 2013). Nevertheless, using shorter fragment of 16S rRNA gene still remains questionable in terms of accuracy of taxonomic classification (Kim et al., 2011; Z. Liu et al., 2007).

2.7.1.3 Limitations of using 16S rRNA for taxonomic identification of bacteria

Massively parallel sequencing is increasingly being used to characterize microbial communities and to provide better understanding of bacterial biodiversity. However, many limitations exist when using 16S rRNA based amplicon sequencing (Poretsky et al., 2014). First, the short reads lengths obtained from the sequencing process might have a

greater noise from sequencing error than sequencing method that produces longer reads such as sanger sequencing (Quince et al., 2011). Secondly, as mentioned earlier, choice of amplification region might lead to differences in inferred species richness (Youssef et al., 2009), and the assessment of OTU cutoffs still needs further validation (Huse et al., 2010). Thirdly, the resolution of 16S rRNA gene between closely related species might not be high enough for taxonomic classification at a species or genus level (Konstantinidis Konstantinos T et al., 2006). Moreover, assessment of diversity based on a single marker gene is prone to overlooking the horizontal gene transfer that may bias the inferred bacterial community composition (Rosselló-Mora & Amann, 2001).

2.7.2 ITS sequencing for characterization of fungal communities

The internal transcribed spacer (ITS) region is the most commonly targeted region for fungal community characterization (Rolf Henrik Nilsson et al., 2009). ITS is a spacer DNA between the small subunit (SSU) rRNA and large subunit (LSU) rRNA in the transcribed region of polycistronic rRNA precursor transcript. There are two ITS regions, one of them being ITS1 that is located between 18S and 5.8S rRNA gene, and the other being ITS2 that is located between 5.8S and 28S rRNA genes (Buchan et al., 2002).

The ITS1 and ITS2 regions have similar variability and length, however, ITS2 is better suited for phylogenetic identification due to the larger number of ITS2 sequences available in the International Nucleotide Sequence Databases (Rolf Henrik Nilsson et al., 2009). Furthermore, the downstream region of ITS2 contains genes that may contribute to further signal of identification since it is a more conserved as shown by its secondary

structure (A. W. Coleman, 2009). Moreover, ITS2 has a greater selection of adjunct data resources available compared to ITS1, which allows identification at higher taxonomic levels (A. W. Coleman, 2009; Selig et al., 2008, p. 2).

Overall, ITS is a highly repeated region in the eukaryotic genome, and the high DNA copy number may enhance the power of microbial characterization (Vilgalys & Gonzalez, 1990). This region also undergoes rapid evolution that promotes genetical uniqueness that permits differentiation among closely related fungal species. ITS is highly variable between closely related species indicating a relatively high discriminating ability for fungi that are phylogenetically close (Baldwin et al., 1995). The ITS fragment size is relatively small (approximately 650 bp) and is possible to amplify and an almost fully sequence using short-red sequencing (Rolf Henrik Nilsson et al., 2009).

2.8 Characterization of microbial and fungal biodiversity

Biodiversity is a concept used for measurement of the biological variety in an ecological system (Fedor & Spellerberg, 2013). Biodiversity can be described using three measures: alpha diversity, beta diversity and gamma diversity. Alpha diversity describes the diversity within a sample (Leung et al., 2018), beta diversity describes the diversity among samples (Bishop et al., 2015), and gamma diversity describes the total species diversity in a given landscape (Arellano & Halffter, 2003).

2.8.1 Alpha diversity

Alpha diversity considers two variables - species richness and evenness. Richness generally refers to a number of different species present in a sample whereas evenness describes how similar are abundances of species in a sample (Poos et al., 2009). Commonly used alpha diversity indices include Chao1 index (Chao, 1984), Shannon diversity index (Lande, 1996; C. E. Shannon, 1948), Simpson index (Simpson, 1949), and Berger-Parker index (Caruso et al., 2008). Chao1 index takes the ratio of singleton to doubleton into account and weights more toward the rare species, however, it only measures species richness, not evenness (Prehn-Kristensen et al., 2018). Shannon diversity index and Simpson index take into account both richness and evenness. Shannon index places greater emphasis on evenness, whereas Simpson index places higher weight on richness (McGarigal & Marks, 1995; Riitters et al., 2000). Inverse Simpson index is often used to represent Simpson index value and show the direct relationship instead of inverse relationship (Simpson) between species diversity and the abundance of the most common species (Sagar & Sharma). Lastly, Berger-Parker index is calculated solely based on evenness (Caruso et al., 2008). In recent studies, Shannon and Simpson indices were most frequently used for estimation of alpha diversity in microbial communities (Keylock, 2005; Nagendra, 2002).

2.8.1.1 Rarefaction as means of OTU normalization

The increased accessibility of sequencing technologies and the introduction of the concept of microbial diversity has given researchers an exciting opportunity to increase

our understanding of microbial ecology. Statistical approaches play a crucial role in studies to understand the patterns underlying the big data. In recent years, rarefaction has become the most commonly used normalization method applied in studies related to microbial diversity (Hughes et al., 2002). Briefly, rarefaction is a way to normalize a sampling effort by randomly subsampling from the original dataset, and calculating the sample richness based on the rarefaction curve (McMurdie & Holmes, 2014). It is represented by the ratio between the number of OTUs generated against the number of unique OTUs obtained. In a sequencing run, well-sequenced sample may have a higher probability for detecting more rare species than poorly sequenced samples and this largely impacts the within-sample diversity or alpha diversity value. Rarefaction is a useful tool for normalization of the sequencing depth to the same level, and expression of community diversity by minimizing the effect of the sequencing effort bias (Curtis et al., 2002).

However, normalization by rarefaction is not suitable in some circumstances. For instance, if species are extremely rare or extremely common or if beta diversity is high, it is likely for rarefaction to miscount alpha diversity by only including a relatively small portion of sequences. Moreover, rarefaction assumes that the taxon occurrence is equivalent to sequencing depth (Hughes & Hellmann, 2005; Hughes et al., 2002). Since it is carried out by randomized subsampling, rarefaction does not reveal the actual number of true richness in a sample (Curtis et al., 2002).

2.8.2 Beta diversity

There are three commonly used parameters for beta diversity estimation. Bray-Curtis dissimilarity is based on the species abundance (Harrison et al., 2011), Jaccard distance is calculated based on the presence or absence of individual species (Harrison et al., 2011), and UniFrac estimates the phylogenetic distance among identified OTUs (C. Lozupone & Knight, 2005). The unweighted UniFrac metric is based solely on the phylogenetic distances (Jianjun Wang et al., 2013), while the weighted UniFrac measures also the presence or absence of species and their abundance (C. A. Lozupone et al., 2007). Most of the published amplicon-based metagenomic studies calculate beta diversity based on the weighted (quantitative) or unweighted (qualitative) UniFrac metric to estimate underlying phylogenetic relationships among identified microbial taxa.

2.8.2.1 Characterization of microbial communities using beta diversity measures

Multivariate statistical approaches allow for identification of microbiota clustering patterns based on the beta diversity measure. This allows for visualization of the effects of biotic and abiotic factors on the microbiota composition (Barberán et al., 2012). Beta diversity based analysis showed that soil microbiota can be significantly affected by environmental stressors such as soil warming, and exposure to copper (Rocca et al., 2019). It also helped to reveal the diversity of microbiota in fermented vegetable facility, so researchers can identify different composition of bacteria in different locations of the processing plant (Einson et al., 2018). Visualization of beta diversity is enabled through various analyses to observe the trend of similarity and difference among samples, and one of the most used technique is Principal Coordinates Analysis (PCoA) (Bokulich

et al., 2013). PCoA is an ordination method that reduces the dimension of original variables in a way that the distance between each point reflects closeness to the original trend (Sogin et al., 2006).

In this thesis, we aimed to use this approach to investigate the similarities and differences among environmental microbiota collected from three different apple packing houses.

2.8.3 Network analysis

High-throughput DNA sequencing allows us to study the co-occurrence of microbial taxa in complex microbial communities. This approach can provide valuable information that allows for further investigation of direct or indirect interactions among bacterial taxa that co-exist or co-exclude each other in a given habitat (Chaffron et al., 2010). Understanding of the microbiota co-occurrence and co-exclusion patterns is a necessary first step in exploration of functional roles that microorganisms play in the environment they occupy (Fuhrman & Steele, 2008).

Network interaction analysis was applied to various studies related to microbiota. Faust et al. constructed a microbial network to show the co-occurrence and co-exclusion relationships and niche specialization of the human microbiota (Faust et al., 2012). Interaction networks are also widely used in the study related to food or food-related systems. For example, Oakley et al. used network analysis to study the co-occurrence of microbiota with foodborne pathogen *Campylobacter* in agricultural animals (Oakley et al., 2013). Moreover, Mounier et al. described the significant yeast-yeast co-occurrence

interactions within a cheese microbial community and their potential role in the ripening of smear cheeses (Mounier et al., 2008). Overall, network analysis of microbiota interactions including co-occurrence and co-exclusion relationship can provide useful insights that may guide experimental design to identify the mechanisms of microbial interactions (Barberán et al., 2012; Proulx et al., 2005). Understanding the interactions among nonpathogenic and pathogenic microorganisms that share the same niche in a food processing environment may provide clues on how to control them.

2.8.4 Prediction of microbiota functional profiles

Understanding of the functional capability of a microbiota in a given environment is another challenge in microbial ecology. Metagenomic sequencing can provide sets of data that can be used to predict detailed metabolic and functional profiles of microbiota, however, shotgun metagenomics is still relatively costly (Langille et al., 2013), hence amplicon sequencing is still broadly used. Amplicon sequencing is based on one or few marker gene(s) and it can be used for characterization of bacteria taxonomic composition in a given sample. However, sequencing data based on marker gene sequencing does not provide direct information about microbiota gene content that would reflect their metabolic or functional capacity (Langille et al., 2013). As a solution to this limitation, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) has been developed that combines taxonomic information obtained through 16S rRNA amplicon with information about the gene content of genomes of representative microbial species that had been previously whole genome sequenced.

PICRUSt is a well-validated and widely used computational tool for functional capacity prediction of microbial communities based on marker genes (Langille et al., 2013; Lyons, Turnbull, Dawson, & Crumlish, 2017). A collection of fully characterized bacterial genomes that is used as a reference for functional characterization is comprised in a PICRUSt database, and marker gene (16S rRNA for bacteria and archaea) is used for taxonomic characterization of targeted microbiota samples. The predictions of metabolic potential is carried out based on the sequence similarity and phylogenetic relationships between characterized full or draft genomes and the corresponding 16S rRNA data (Langille et al., 2013) (Figure 2.4).

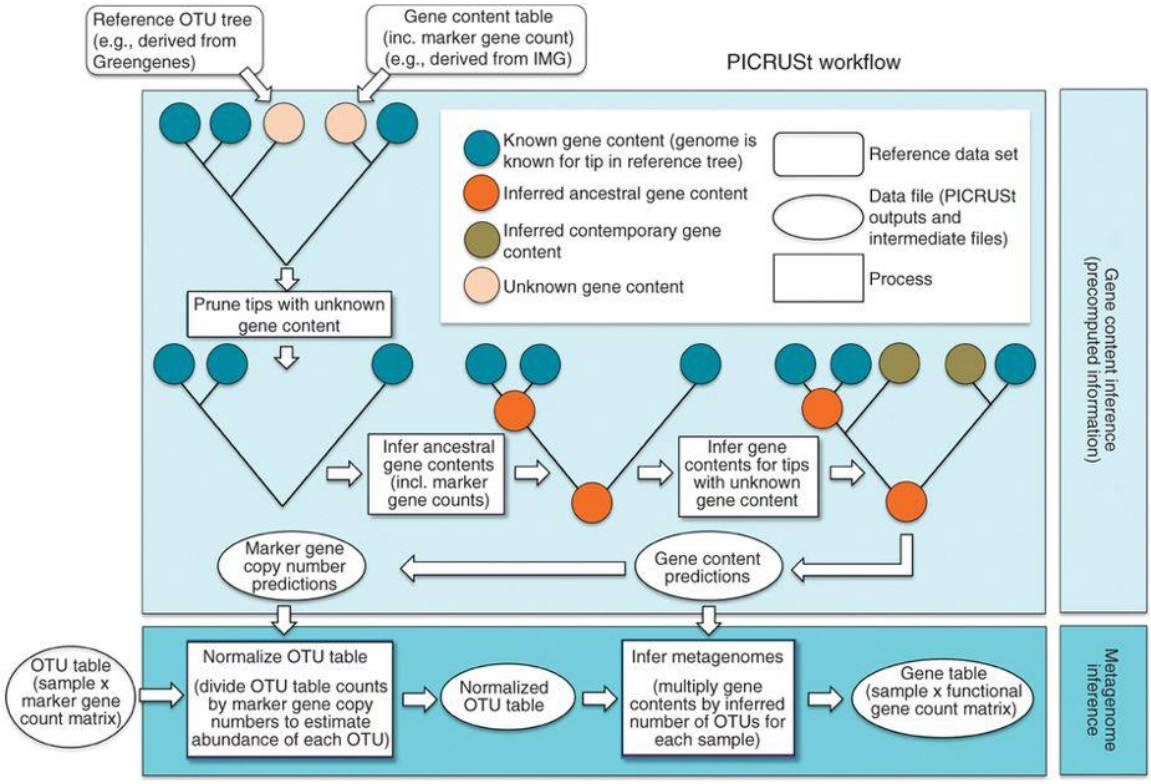

Figure 2. 4**:** PICRUSt functional profile prediction based on a marker gene (Langille et al., 2013).

PICRUSt is widely used as a functional prediction tool (Hartman et al., 2017; Reed et al., 2017; Ren et al., 2017; Wilkinson et al., 2017, 2018) and has been applied to investigate factors such as how the intake of dietary iron alters the structure and function properties of gut microbiota (Reed et al., 2017). Environmental microbiota studies have also utilized this tool to predict functional changes in sponge microbiota in a response to environmental stressors (Lesser et al., 2016). Furthermore, the relationship between environmental factors and microbial biofilm metabolism have been investigated in glacier-fed streams (Ren et al., 2017).

PICRUSt is a rapid way of functional profiling of microbiota, however, there are limitations associated with the use of this tool. Firstly, it requires all OTUs assigned to a pre-defined reference based on Greengene 16S rRNA database. Other databases such as SILVA currently cannot be used for the functional profile prediction (Douglas et al., 2018). Secondly, the prediction is fully dependent on the similarity between marker genes and the genes from reference genomes, and the reference genomes are usually fewer and less diverse compared to the genomes from microbiome samples (Y. Yang et al., 2016). Anothr major limitation of PICRUSt is that the prediction accuracy is depend on the accuracy of gene annotations, however, microbial gene annotation usually has a low accuracy level shown by the previous researches. Therefore, the interpretation of prediction needs to be carefully validated (Langille, 2018). In conclusion, PICRUSt is an effective tool for bacterial community functional profile prediction, but the inferences need to be further (experimentally) validated. To date, no study has used this approach to predict the functional profiles of the native microbiota in food processing environments.

2.9 Conclusion and research objectives

Fresh fruit and vegetable consumption has been increasing due to their health benefits (Lee-Kwan et al., 2017; Vereecken et al., 2015). However, recent outbreaks and recalls associated with this common food category have raised public and regulatory food safety concerns since these foods are commonly consumed raw or minimally processed. In the U.S. alone, the estimated cost of recalls due to *L. monocytogenes* contamination may be between $1.2 billion to $2.4 billion annually. And the estimated medical cost or premature death loss related to listeriosis is approximately $2.3 billion a year (Ivanek et al., 2004).

Previous studies suggest that fresh produce preharvest environment could be a source of *L. monocytogenes*, however, the risk of contamination in the apple or tree fruit orchard has not been studied. Previous research has also revealed that *L. monocytogenes* can survive on fresh whole apples and interact with other microorganisms that may promote or inhibit its growth. However, no study to date has investigated the microbial composition and species co-occurrence in apple packing houses. In addition, the relationship between *L. monocytogenes* and background core microbiota in the processing environment has not yet been determined.

The purpose of this study was therefore to determine the extent to which associations exist between *L. monocytogenes* occurrence and overall microbial diversity in the built apple packing house environment. A better understanding of the relationship between *L. monocytogenes* persistence and the packing house microbiota could be useful

for developing novel control interventions to lower the risk for apple contamination to occur.

The objectives of this study were to:

1. Determine the occurrence and levels of *L. monocytogenes* on selected non-food contact surfaces in three packing facilities;
2. Determine the composition of bacterial and fungal communities in these packing facilities through high throughput 16S rRNA and ITS2 amplicon sequencing and analyze the data for potential associations between the microbiota composition and occurrence of *L. monocytogenes*.

Chapter 3

Built environment microbiota is associated with occurrence of *Listeria monocytogenes* in three tree fruit processing facilities

3.1 Abstract

Multistate foodborne outbreaks and recalls of apples and apple products contaminated with *Listeria monocytogenes* demonstrate the need for improved pathogen control in the apple supply chain. Apple processing facilities have been identified in the past as potential sources of persisting *L. monocytogenes* contamination. In this study, we sought to understand the composition of microbiota in built apple processing environments and its association with the occurrence of the foodborne pathogen *L. monocytogenes*.

Analysis of 117 samples collected from three apple packing facilities (F1, F2 and F3) showed that facility F2 had a significantly higher *L. monocytogenes* occurrence compared to F1 and F3 ($p < 0.01$). The microbiota in facility F2 was distinct compared to facilities F1 and F3 as supported by the mean Shannon index for bacterial and fungal alpha diversities that was significantly lower in F2, compared to F1 and F3 ($p < 0.01$). Microbiota in F2 was uniquely predominated by bacterial family Pseudomonadaceae and fungal family Dipodascaceae.

The composition and diversity of microbiota and mycobiota present in the investigated built food processing environments may be indicative of persistent contamination with *L. monocytogenes*. These findings support the need for further

investigations of the role of the microbial communities in the persistence of *L. monocytogenes* to support the optimization of *L. monocytogenes* control strategies in the apple supply chain

3.2 Background

Listeriosis is a foodborne infectious disease caused by *Listeria monocytogenes*. Listeriosis has 20 to 30% fatality rate in high-risk groups, such as elderly, pregnant woman and immunocompromised individuals (Swaminathan & Gerner-Smidt, 2007). *L. monocytogenes* infections have been historically associated with outbreaks traced back to ready-to-eat meat products (Gombas et al., 2003) and unpasteurized raw milk (Lovett, Francis, & Hunt, 1987). However, in recent years, an increased number of listeriosis outbreaks have been linked with contaminated fresh produce (CDC, 2018; Zhu, Gooneratne, & Hussain, 2017). In 2014, a multistate outbreak of listeriosis traced back to contaminated prepackaged caramel apples caused 34 hospitalizations and 7 deaths (CDC, 2018). In 2017, three more cases of listeriosis associated with prepackaged caramel apples were reported (Marus, 2019). Several recalls of sliced apples contaminated with *L. monocytogenes* have raised broader concerns about the safety of apples, primarily when further processing provides suitable conditions for *L. monocytogenes* growth (Glass et al., 2015; Government of Canada, 2015). Increased food safety scrutiny has led to enhanced monitoring of *L. monocytogenes* in tree fruit packing and processing facilities, and there is a need to investigate factors that may play a role in the establishment and persistence of *L. monocytogenes* in built environments in the apple supply continuum.

A number of studies have investigated the effects of commonly used sanitizers on the reduction of *L. monocytogenes* and on its ability to adapt to these antimicrobial treatments (Chavant et al., 2004; J. Lundén, Autio, Markkula, Hellström, & Korkeala, 2003; Pan et al., 2006; Taormina & Beuchat, 2002). These studies provided valuable insight into the ability of sanitizers to inhibit the growth of *L. monocytogenes* in monocultures (Beuchat, Adler, & Lang, 2004; Chavant et al., 2004); however, they failed to adequately model the complex biotic environmental conditions found in post-harvest food processing built environments (Bokulich, Lewis, Boundy-Mills, & Mills, 2016; Doyle, O'Toole, & Cotter, 2017). It is becoming increasingly evident that microbiota found in food production and processing environments plays a role in pathogen survival and persistence and that microbiota needs to be taken in consideration when assessing the effectiveness of pathogen control strategies (Doyle et al., 2017).

Interspecies interactions between *L. monocytogenes* and other microorganisms that make up the food processing environment microbiota have been shown to alter the ability of *L. monocytogenes* to survive and colonize facilities (Giaouris et al., 2015). For example, *Pseudomonas* spp. commonly found in food processing environments (Hassan et al., 2004),has been found to have a positive effect on *L. monocytogenes* attachment on the stainless steel surfaces (Hassan et al., 2004). In contrast, *Staphylococcus sciuri* was shown to decrease *L. monocytogenes* biofilm formation on stainless steel surfaces (Leriche & Carpentier, 2000). Carpentier and Chassaing (Carpentier & Chassaing, 2004) demonstrated that among 29 bacteria isolated from the dairy and meat processing environment, 4 strains promoted *L. monocytogenes* growth in the resulting biofilm when

co-cultured with *L. monocytogenes* whereas 16 strains suppressed the growth of *L. monocytogenes*. Although the exact mechanisms underlying these biological interactions and phenotypic outcomes are yet to be elucidated, competition for nutrients and production of anti-*Listeria*l secondary metabolites have been proposed as important microbiota-shaping factors (Kunze et al., 2010; Leriche & Carpentier, 2000). One of the important steps toward gaining a better understanding of microbial interactions in microbial communities is the characterization of environmental microbiota in food production and processing facilities and investigation of associations and co-occurrence of *L. monocytogenes* and other members of the microbiota.

Amplicon sequencing has revolutionized the characterization of microbial communities, not only in human medicine and ecology but also in the food industry where information about microbiota dynamics can enhance our ability to answer applied questions related to food safety and quality. Microorganisms such as *P. psychrophila*, *Pseudomonas sp*., *Klebsiella sp*., *K. oxytoca* and *A. hydrophila* have been identified through 16S rRNA sequencing as the dominant species in *L. monocytogenes*-positive drains located in dairy, meat, peanut butter, and spice processing plants, suggesting that they might facilitate *L. monocytogenes* biofilm formation through interspecies interactions (Y. Liu et al., 2016). Moreover, studies have been carried out in dairy and meat processing plants, where associations have been found between the indoor bacterial communities and the presence of *L. monocytogenes* (E. M. Fox, Solomon, Moore, Wall, & Fanning, 2014; Stellato et al., 2016). Food processing facilities that serve as an intermediate between the pre-harvest, raw ingredient, and retail distribution chain have

been identified amongst the main potential sources of pathogen contamination (Berger et al., 2010; Beuchat, 1996; Ho, Lappi, & Wiedmann, 2007; Kabuki, Kuaye, Wiedmann, & Boor, 2004). It is therefore critical to establish a baseline understanding of microbial diversity in food processing environments and to investigate the associations between microbial community composition and occurrence of *L. monocytogenes* in these environments. This knowledge can be used to develop targeted microbiota manipulation strategies for the development of improved foodborne pathogen control strategies. There is only limited data available on the composition of microbial communities in built produce processing environments (Gu et al., 2019). To the best of our knowledge, no published studies have reported the relationship between apple packing house microbiota composition and the presence of *L. monocytogenes* in these environments to date. We, therefore, utilized 16S rRNA V4 and ITS2 amplicon sequencing coupled with *L. monocytogenes* enrichment to elucidate the associations between the composition, diversity, and predicted functional profiles of the built environment microbiota in three apple packing facilities to provide new knowledge enabling improvement of food safety.

3.3 Material and Methods

3.3.1 Study design

Occurrence of *Listeria monocytogenes* and characterization of microbiota within three apple packing houses in the northeast U.S. was monitored through the fruit harvesting and packing season. At each facility, nonfood-contact (Zone 3) environmental

samples were collected from the floor under a conveyor system with rolling brushes that transported fruit through successive washing, drying, and waxing processes, as outlined below. These specific locations were selected for sampling because preliminary data 2016/17 showed a higher occurrence of *L. monocytogenes* in these locations compared to other locations in the packing houses. The preliminary data established that a sample size of 117 would is needed to identify the prevalence of *L. monocytogenes* with a 7.25% precision and 95% confidence (Naing, Winn, & Rusli). Therefore, a total of 117 samples were collected twice a month over 13 sampling periods between November 2017 and April 2018.

3.3.2 Sample collection

Non food-contact-surface environmental areas (40 cm by 40 cm) were sampled by swabbing with pre-moistened sponges (3M) with a combination of ten horizontal and 10 vertical strokes. Two adjacent duplicate areas were sampled at each of the three areas (washing, drying, waxing) under the conveyor belt (Fig. 1). One of the duplicate samples was used for *Listeria* spp. enrichment, isolation and identification, and the other for microbiota characterization using 16S rRNA V4 and ITS2 Illumina sequencing. Samples for *Listeria* spp. isolation and identification were collected with 3M hydrated sponges pre-moistened with 10 mL D/E neutralizing buffer (3M) to enhance the survival of *Listeria* spp. through the neutralization of a broader range of sanitizers (FDA, 2017). The samples for microbiota characterization were collected using 3M hydrated sponges with

just 10 mL of neutralizing buffer. All swab samples were stored in a cooler on the ice during transportation to the laboratory and were processed on the same day.

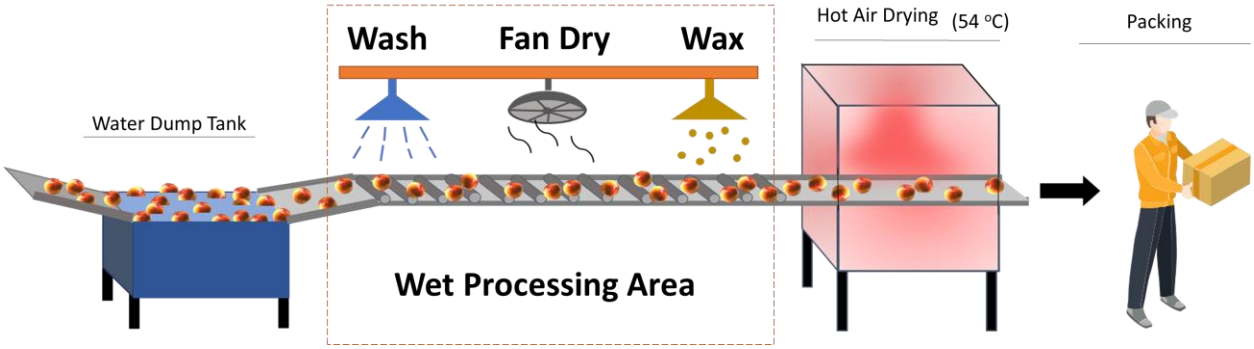

Figure 3. 1**:** Apple processing workflow representative of monitored apple packing facilities. Samples were collected in the wet processing area under the rotating brush conveyor belt, that consisted of washing, drying, and waxing area.

3.3.3 *Listeria monocytogenes* enrichment and isolation.

Detection of *Listeria* spp. was conducted following a modified Food and Drug Administration Bacteriological Analytical Manual (FDA BAM) protocol for the detection and enumeration of *L. monocytogenes* (FDA, 2017). Briefly, 90 ml of Buffered *Listeria* Enrichment Broth (BLEB, Oxoid) were added to each test sample and control sample, followed by manual homogenization by hand-massaging the sponge for 15 seconds. *L. monocytogenes* strain F2365 (Chen & Knabel, 2007) and *Listeria innocua* strain PS00298 were used as positive and negative controls in every sample processing batch, respectively, to compare their morphology with the morphology of putative *Listeria* spp. isolated on selective agars as well as with the resulting PCR product separated using gel electrophoresis. A sterile sealed sampling sponge from the same production batch served as an additional negative control to ensure that the used batch of sampling sponges was

not contaminated with *L. monocytogenes*. For the enrichment, 400 µl of BLEB supplement SR0149 (Oxoid) were added to each test sample and controls after 4 hours of pre-enrichment in BLEB at 30 ºC. After adding the supplement, samples were further incubated for 44 ± 2 hours at 30 ºC. Upon completed incubation, a loopful of each enrichment was streaked onto Agar *Listeria* Ottavani & Agosti (ALOA, BioRad Laboratories Inc.) and RAPID' *L mono* (BioRad Laboratories Inc.) selective differential agars, and incubated for 48 hours at 37 ºC. After incubation, one typical colony was collected from each, ALOA and RAPID *L' mono* agars, and streaked for isolation on Trypticase Soy Agar with Yeast Extract (TSAYE). Streaked plates were incubated for 24 hours at 37 ºC and isolated colonies were used for PCR-based *L. monocytogenes* confirmation.

3.3.4 *Listeria monocytogenes* identification and cryopreservation.

One isolated colony per TSAYE plate was selected, inoculated in the Trypticase Soy Broth with Yeast Extract (TSBYE) and incubated at 30 ºC for 24 hours. One milliliter of each overnight culture was centrifuged at 15,000 g for 15 minutes, washed with DNase free water and centrifuged again at 15,000 g for 15 minutes. The cell pellets were resuspended in 50 µl of DNase free water and heated for 10 minutes at 95 °C to lyse the cells. Lysates were then centrifuged at 15,000 g for 10 minutes to remove the cell debris. Supernatants containing DNA template were stored at -20 °C until further use. Two microliters of DNA template were used in a PCR reaction with primers targeting genes *iap* (specific for *Listeria* spp.) and *lmo2234* (specific for *L. monocytogenes*) to

confirm *the L. monocytogenes* species (Chen & Knabel, 2007). *L. monocytogenes* strain F2365 and *Listeria innocua* strain PS00298 served as positive controls and the nuclease-free water was used as a negative control. The following thermal cycling conditions were used: initial denaturation at 95 °C for 15 minutes, 15 cycles of denaturation at 94 °C for 1 minute, annealing at 55 °C to 51 °C for 1 minute with a touch down of 3 cycles per temperature, extension at 72 °C for 1 minute. The following 15 cycles started with denaturation at 94 °C for 1 minute, annealing at 50 °C for 1 minute, extension at 72 °C for 1 minute and the final extension at 72 °C for 8 minutes (Chen & Knabel, 2007). Successful PCR amplification was confirmed by gel electrophoresis using 2% agarose gel (Invitrogen). A band between 1450 bp to 1600 bp (for *iap*) and a band at 420 bp (specific for *lmo2234*) were expected in samples positive for *Listeria* spp. or *L. monocytogenes*, respectively. Isolates that were confirmed as *Listeria* spp. or *L. monocytogenes* were re-streaked on TSAYE and grown overnight in TSBYE. Overnight cultures were supplemented with 20% glycerol and stored in -80 °C freezer.

3.3.5 Quantification of *Listeria* using the most probable number assay.

Most Probable Number (MPN) assay for *L. monocytogenes* was carried out on 18 samples collected in two samplings conducted in March 2018 to determine the level of *L. monocytogenes* contamination, using the enrichment media described above. Briefly, 90 ml of BLEB were added to a bag containing a sponge sample and manually homogenized. Six serial ten-fold dilutions of the homogenate were prepared in BLEB. Three-tube MPN assay was carried out using 1, 0.1, 0.01, 0.001 and 0.0001 ml of each

sample with BLEB added to a total volume of 10 ml. 40 µl of BLEB supplement were added to each tube after 4 hours of incubation at 30 ºC. Supplemented samples were further incubated for 44 ± 2 hours at the same temperature. After 48-hour incubation, a loopful of each dilution enrichment was streaked onto ALOA and RAPID' *L. mono* agar plates and incubated at 37 ºC for 24 to 48 hours, until obtaining visible colonies. Colonies appearing blue-green with an opaque halo on ALOA agar, and black with a yellow background on RAPID' *L. mono* agar were considered as putative *L. monocytogenes*. The MPN of *L. monocytogenes* per sponge sample was determined using an MPN calculator provided in the Excel file available for download in "BAM: Detection and Enumeration of *Listeria monocytogenes*" (FDA, 2017).

3.3.6 Total DNA extraction for microbiota and mycobiota analysis.

Each environmental sponge sample collected for microbiota characterization was homogenized with 50 ml of phosphate buffer containing 0.9% NaCl in a stomacher, for 7 minutes at 230 rpm. Fifty milliliters of the homogenate were transferred to a sterile 50 ml conical tube and centrifuged at 11,000 g and 4 ºC for 20 minutes (Beckman Coulter, Avanti J-26 XPI) (Tringe et al., 2008). After centrifugation, supernatants were discarded and pellets were stored at -80 °C until DNA extraction. DNA was extracted from approximately 0.25 g of each sample using DNeasy PowerSoil DNA extraction kit (Qiagen) following manufacturer's protocol. Approximately 0.25 g of a sterile sponge was also sampled and used as a negative control to confirm the absence of microbial DNA contaminants on the sterile sponge. DNA extracted from the sponge was processed

following the same protocol as described below for other samples. The concentration of DNA in each test sample and in the control sample was determined both spectrophotometrically using NanodropOne (Thermo Scientific) and fluorometrically using Qubit 3 (Invitrogen) and Qubit dsDNA High Sensitivity Assay Kit. DNA samples were stored at -80 ºC until further use.

3.3.7 16S rRNA V4 and ITS2 sequence amplification.

Bacterial and fungal community composition was determined by targeted metagenomic sequencing of the PCR-amplified V4 domain of the 16S rRNA gene and the internal transcribed spacer 2 (ITS2) sequences, respectively. Briefly, V4 region of the 16S rRNA gene sequence was amplified using KAPA HiFi HotStart ReadyMix (Kapa Biosystems), a forward primer 505F-v2 (TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GTG YCA GCM GCC GCG GTA A), and a reverse primer 806R-v2 (GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGG ACT ACN VGG GTW TCT AAT) (Thompson et al., 2017). PCR thermal cycling for amplification of the 16S rRNA gene V4 region was conducted as follows: initial denaturation at 95 °C for 3 min, 29 cycles of denaturation at 98 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and the final extension at 72 °C for 5 min, and final hold at 4 °C. Fungal ITS loci were amplified using forward a primer ITS4F (TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG GAA CGC AGC RAA IIG YGA) and a reverse primer ITS9R (GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GTC CTC CGC TTA TTG ATA TGC)(Nordberg et al., 2014). PCR conditions for ITS amplification were

as follows: initial denaturation at 98 °C for 5 minutes, 30 cycles of denaturation at 95 °C for 45 s, annealing at 50 °C for 60 s, extension at 72 °C for 60 s, and the final extension at 72 °C for 5 min, and the final hold at 4 °C. PCR amplicons were visualized by running gel electrophoresis using a 2% agarose gel to confirm successful amplification of target sequences.

3.3.8 Amplicon library preparation and amplicon sequencing.

Amplicon libraries were prepared based on Illumina's 16S Metagenomic Sequencing Library Preparation protocol (Illumina, 2013). 16S rRNA V4 and ITS2 PCR amplicons were barcoded with unique combinations of i7 and i5 index adaptors (Integrated DNA Technologies) in a second-step PCR using following thermal cycling conditions: initial denaturation at 95 °C for 3 min, 8 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 5 min; final hold was at 4 °C. Barcoded PCR amplicon libraries were purified twice with AmPure XP beads (Beckman Coulter) and then normalized using Mag-Bind EquiPure Library Normalization Kit (Omega Bio-tek) following manufacturer's protocol. Concentrations of a subset of normalized libraries were verified using a high sensitivity double-stranded DNA kit with Qubit 3. Libraries were then pooled in equal volumes of 4 µl. The distribution of pooled library fragment sizes was verified using Bioanalyzer and its concentration using qPCR. Estimated amplicon lengths of 359 bp and 425 bp were used in the calculation of molar concentration and normalization. The library pool was denatured by diluting 5 µl of 4 nM library pool with 5 µl freshly prepared 0.2 N NaOH.

The denatured library was diluted with a pre-chilled HT1 buffer to 7.5 pM with 10% PhiX internal control library. A total of 600 µl of denatured library spiked with PhiX was loaded onto the Illumina Miseq flow cell. Five hundred cycle V2 Illumina sequencing kit was used for 250 bp paired-end sequencing in two Illumina MiSeq sequencing runs. Sequencing reads were deposited in NCBI SRA under BioProject PRJNA527988.

3.3.9 Sequence analyses and OTU normalization.

Sequences were analyzed with Mothur v1.39.5 following protocols described in Schloss et al. (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013; Schloss et al., 2009) with default settings unless otherwise noted. Paired end sequence reads were assembled into contigs. Contigs shorter or longer than 292 bp for 16S rRNA V4 bacterial sequences and longer than 350 bp for ITS2 fungal sequences were discarded. The remaining reads were aligned against SILVA database (Quast et al., 2013) for 16S rRNA V4 region bacterial sequences, and UNITE database (Kõljalg et al., 2013) for ITS2 fungal sequences. Chimera were detected and discarded using UCHIME algorithm and the remaining sequences were assigned to taxonomy using SILVA or UNITE databases. OTUs were calculated using opticlust with 97% similarity threshold. All 16S rRNA sequences were rarefied to 4,501 and ITS sequences were rarefied to 5,232 randomly sampled OTUs. Rarefied sequence cutoffs were chosen based on the lowest reads obtained for microbiota and mycobiota samples, in order to include all samples in downstream comparative analyses.

3.3.10 Diversity of microbiota and mycobiota.

Alpha and beta diversity indices were calculated based on rarefied OTUs. For alpha diversity, Shannon and Inversed Simpson indices were calculated for both bacterial and fungal communities. For beta diversity, the weighted UniFrac distance between either bacterial or fungal communities was calculated in R using package Phyloseq (McMurdie & Holmes, 2013). Principal Coordinates Analysis (PCoA) was used to visualize the beta diversity of microbiota with reduced dimensionality. Principal coordinates were plotted using R package ggplot2 (Wickham, 2016). Chi-square and Fisher's exact tests with Bonferroni correction were used to test the significance of differences in *L. monocytogenes* occurrence among apple packing facilities. Pairwise permutational multivariate analysis of variance (PERMANOVA) test was carried out using package PairwiseAdonis (Arbizu, 2019, p. 3) and Bary-Curtis dissimilarity matrices were used to test the significance of differences in microbiota and mycobiota composition. Pairwise comparisons were carried out by sampling month, a processing section, and facility.

3.3.11 The co-occurrence of microbial and fungal taxa.

Network analyses were carried out to identify significantly co-occurring bacterial families among samples collected from three apple packing facilities. Networks were constructed using Cytoscape v3.7.1 (P. Shannon et al., 2003) with CoNet version 1.1.1 beta plug-in (Faust & Raes, 2016). Rarefied 16S rRNA OTU table was condensed to family level taxa and used as an input matrix. Network analysis was conducted based on

microbial family occurrence for each individual facility as well as for all three facilities combined. Co-occurrence of specific families was determined using Pearson and Spearman correlations, mutual information, and Bray Curtis and Kullback-Leibler dissimilarity indices. Row-shuffle randomization and bootstrap method were used to minimize composition-induced false correlations. The resulting p-values were merged using Brown's method and corrected by Benjamini-Hochberg multiple comparison corrections. Interactions were visualized using yfiles layout algorithms plug-in application (Wiese, Eiglsperger, & Kaufmann, 2004).

3.3.12 Prediction of microbiota's functional profiles.

Raw 16S rRNA sequence reads were re-analyzed using Mothur pipeline outlined above, only this time in conjunction with Greengenes 16S rRNA database version13.8 (DeSantis et al., 2006) since Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) (Langille et al., 2013) required the use of this database. The biom file resulting from Mothur analysis, and containing OTU and taxonomy information was used as an input file for PICRUSt analysis using online Galaxy-based version provided by Hutlab (Huttenhower). Briefly, OTU table was first corrected for 16S rRNA copy number by dividing OTU counts by the number of 16S rRNA marker gene copy to obtain an abundance estimation for each OTU. Each Kyoto Encyclopedia of Gene and Genomes (KEGG) (Kanehisa & Goto, 2000) ortholog (KO) was then multiplied by the normalized OTU abundance for metagenome prediction. KO tier3 was assigned for functional prediction and tire 1 assignments were used for the

categorization of functional profiles for each facility. The output data table contained summed predicted KO functional gene abundance per metagenome sample.

3.4 Results

3.4.1 Samples collected from facility F2 higher occurrence and level of *L. monocytogenes* compared to those collected from F1 and F3.

A total of 117 environmental samples were tested for the presence of *L. monocytogenes* using the FDA BAM enrichment protocol. Out of 39 samples collected in each facility, *L. monocytogenes* culture was isolated and confirmed in 11, 39 and 16 samples from facilities. F1, F2, and F3, respectively (Figure 3.2). All samples in facility F2 were positive for *L. monocytogenes* using the culturing method, while 11 (28.2%) and 16 (41.0%) samples were positive in facilities F1 and F3, respectively ($p < 0.001$). There was no significant difference in *L. monocytogenes* occurrence when comparing washing, fan-drying and waxing sections ($p = 0.112$).

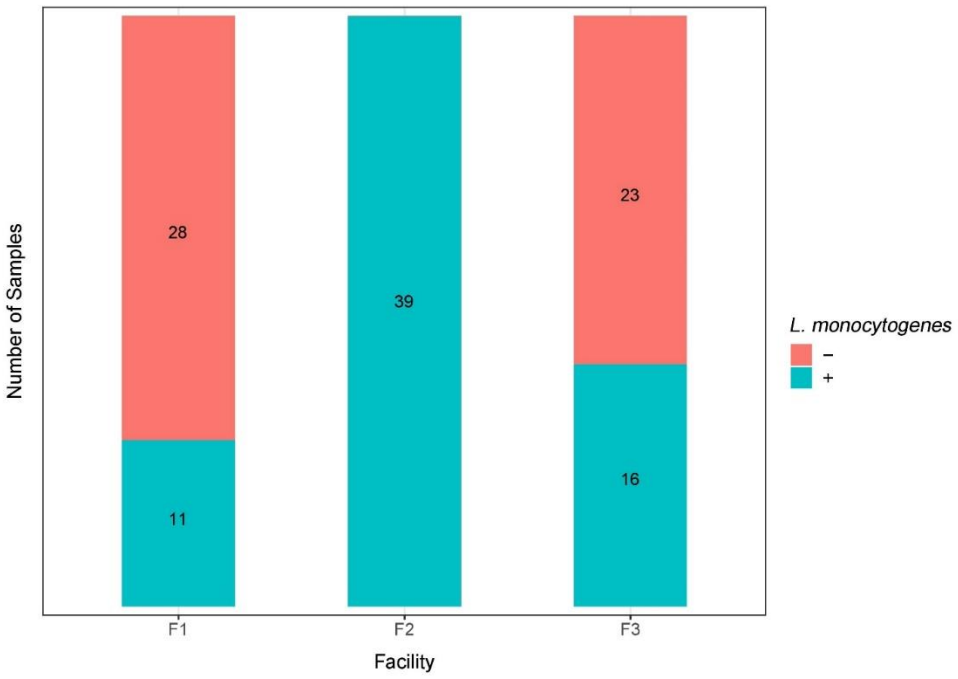

Figure 3. 2: Summary of the presence (+) and absence (-) of *Listeria monocytogenes* in environmental samples collected from three apple packing facilities F1, F2, and F3 over the sampling period (November 2017 – April 2018), as determined using an enrichment protocol.

Quantification of *L. monocytogenes* in a subset of samples collected on two days in March 2018 indicated high-level contamination of F2 at both sampling times. Samples collected underneath the conveyor belt in washing, fan-drying and waxing sections of the facility F2 contained an average of 3.51, 4.18, and 4.87 $\log_{10}$MPN/sponge, while an average of 1.50, 0.82, and 2.00 $\log_{10}$MPN/sponge were found in respective areas in facility F3. No *L. monocytogenes* was detected in samples collected from facility F1 in the two sampling time points (Table 3.1). Samples collected from three facilities had significantly different levels of *L. monocytogenes* ($p < 0.001$), with samples collected

from F2 having higher-level of contamination compared to those collected from F1 and F3.

Table 3. 1: Summary of MPN results for quantitative analysis of *L. monocytogenes* in samples collected from three apple packing facilities at two time points in March 2018.

Log MPN/ sponge sample	Facility F1[c]			Facility F2			Facility F3[c]		
	wash	dry	wax	wash	dry	wax	wash	dry	wax
Trial 1[a]	-	-	-	3.87	5.03	4.38	1.36	-	1.36
Trial 2[b]	-	-	-	3.15	3.32	5.36	1.63	1.63	2.63
Average	-	-	-	3.51	4.16	4.87	1.5	0.82	2.00

[a]Four dilutions (10^{-2},10^{-3},10^{-4},10^{-5}) and the original sample homogenate (10^{-1}) were used in MPN calculation. Each dilution was tested in triplicates.

[b]Five dilutions (10^{-2},10^{-3},10^{-4},10^{-5}, 10^{-6}) and the original sample homogenate (10^{-1}) were used in MPN calculation. Each dilution was tested in triplicates.

[c] – indicates that no *L. monocytogenes* colonies were detected.

3.4.2 16S rRNA and ITS2 were rarefied to the lowest OTU count prior to downstream analyses.

In the first sequencing run, a total of 8,298,240 16S rRNA V4 reads and 8,688,706 ITS2 reads were produced. Samples that were sequenced with fewer than 5,000 reads were re-sequenced in a second sequencing run. In the second sequencing run, 7,458,554 16S rRNA reads and 3,485,400 ITS2 reads were produced. For16S rRNA sequences, a minimum, median and maximum number of unique OTUs identified was 4,501, 28,056 and 168,219, respectively, and for ITS2 sequences, a minimum, median and maximum numbers of identified unique OTUs were 5,323, 17,882 and 350,415.

Rarefaction curves were plotted using the number of OTUs which represented the sequence sample size, against the number of unique OTUs which represented species richness (Figure 3.3). All three facilities generally had a similar sequence sample size, however, richness in samples from facility F2 16S rRNA reached saturation at fewer reads compared to facilities F1 and F3, indicating lower microbial diversity in F2. For ITS2 OTUs, samples from facility F2 had a substantially higher numbers of assembled OTUs compared to samples from F1 and F3. The rarefaction curves for samples from facilities F1 and F3 did not reach saturation, indicating that considerable microbial diversity of low-abundant taxa has not been discovered at a given sequencing depth. Based on the rarefaction curves and our interest in high-abundant taxa that were hypothesized to play a major role in the microbial ecology of monitored environments, we rarefied samples to the lowest number of OTUs found among sequenced samples (i.e., N=4,501 for 16S rRNA V4 sequences and N=5,323 for ITS2 sequences).

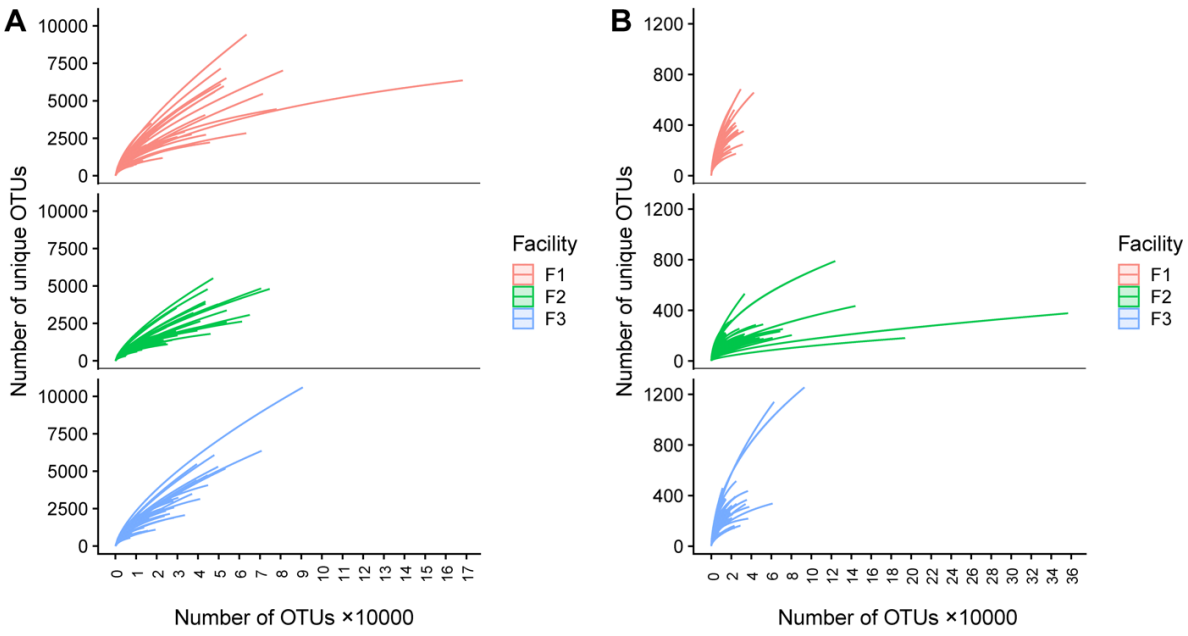

Figure 3. 3**:** Rarefaction curves for 16S rRNA V4 (A) and ITS2 (B) sequences of individual samples were plotted using the number of OTUs that represent the sequence sample size and the number of unique OTUs that indicates the species richness. Each curve is color-coded based on the facility in which the sample was collected.

3.4.3 Alpha diversity of bacterial communities was significantly different in samples collected from facility F2 compared to those collected from facilities F1 and F3.

Microbial and fungal composition of environmental samples collected from three facilities were characterized using rarefied 16S rRNA V4 and ITS2 amplicon sequencing. Alpha diversity indices were visualized using violin plots (Figure 3.4) showing microbiota and mycobiota diversity within each individual facility. Significant differences in bacterial alpha diversity were observed among microbiota of samples collected in facilities F1, F3, and F2. Pairwise t-test comparison showed no significant

difference in microbial alpha diversity determined based on Inverse Simpson index (Figure 3.4A) between facilities F1 and F3 ($p = 1.00$), while significant difference was observed between diversity of microbiota from facilities F1 and F2 ($p = 1.7\times10^{-5}$), as well as from facilities F3 and F2 ($p = 1.5\times10^{-4}$). Shannon index showed similar trends when comparing the alpha diversity of samples from F1 with samples from F3 ($p = 1.00$). Alpha diversities of samples from F1 ($p = 6.0\times10^{-10}$) and F3 ($p = 1.5\times10^{-9}$) were significantly different from those from facility F2, according to the Shannon index (Figure 3.4B).

Alpha diversity indices for fungal communities were also compared among samples collected in three facilities. Significant differences were identified between samples from facilities F1 and F2 ($p = 7.1\times10^{-6}$) as well as samples from F2 and F3 ($p = 1.6\times10^{-4}$) as indicated by the Shannon index. No significant difference was observed between the alpha diversity of samples from F1 and F3 ($p=1.0$, with Bonferroni correction, Figure 3.4D). Inverse Simpson index, on the other hand, showed no significant difference between samples from F1 and F2 ($p = 0.082$) nor F1 and F3 ($p = 1.00$). A significant difference, however, was observed between F2 and F3 ($p = 0.011$) (Figure 3.4C). Overall, the diversity of facility F2 microbiota was substantially lower compared to microbiota sampled in facilities F1 and F3.

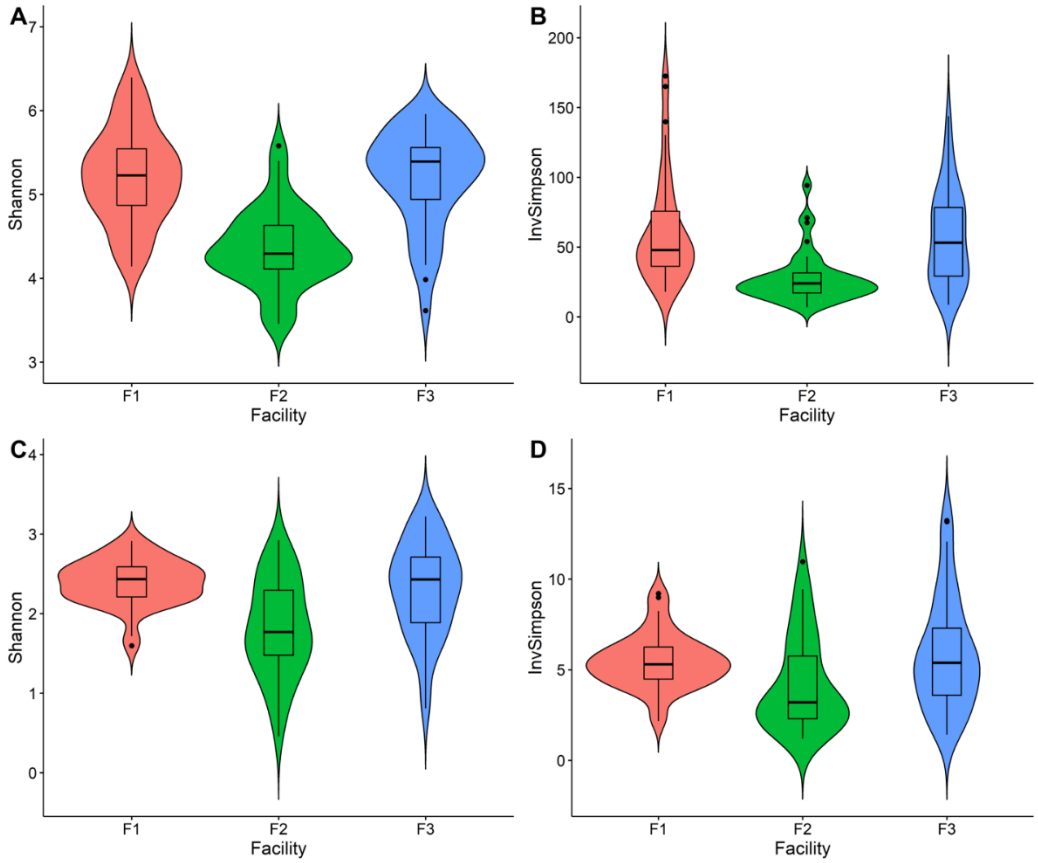

Figure 3. 4: Microbial alpha diversity distributions determined based on rarefied 16S rRNA V4 and ITS2 assembled contigs for samples collected from three packing facilities, F1, F2 and F3. Alpha diversity was measured using Shannon index (A) and Inverse Simpson index (B). Fungal alpha diversity was determined based on rarefied ITS2 assembled contigs, as measured using Shannon index (C) and Inverse Simpson index (D).

3.4.4 Environmental microbiota in facility F2 significantly differed from environmental microbiota in facilities F1 and F3.

The beta diversity of bacterial communities among facilities was shown in the PCoA plot (Figure 3.5). Clustering was analyzed using weighted UniFrac metric which incorporates phylogenetic relativeness when calculating distance among 16S rRNA and ITS2 gene sequence. 10.7% and 6.6% variances in bacterial composition were

represented by the PC1 and PC2, respectively. 43.1% and 20.5% variability in the beta diversity were explained by the PC1 and PC2 for mycobiota. Microbiota of samples collected from facility F2 formed a distinct cluster while microbiota of samples collected from facilities F1 and F3 appeared to be more related to each other than to microbiota from F2 (Figure 3.5A). Consistently with distinct F2 microbiota clustering, mycobiota from this facility also appeared to be different compared to that discovered in facilities F1 and F3 (Figure 3.5B). However, unlike microbiota, the mycobiota found in samples from facilities F1 and F3 appeared to be less similar (Figure 3.5B). In order to further explore the microbial composition that contributed to the distinct clustering of microbiota in the PCoA plot, we investigated the taxonomic composition of collected samples in three individual facilities using PERMANOVA statistical analyses.

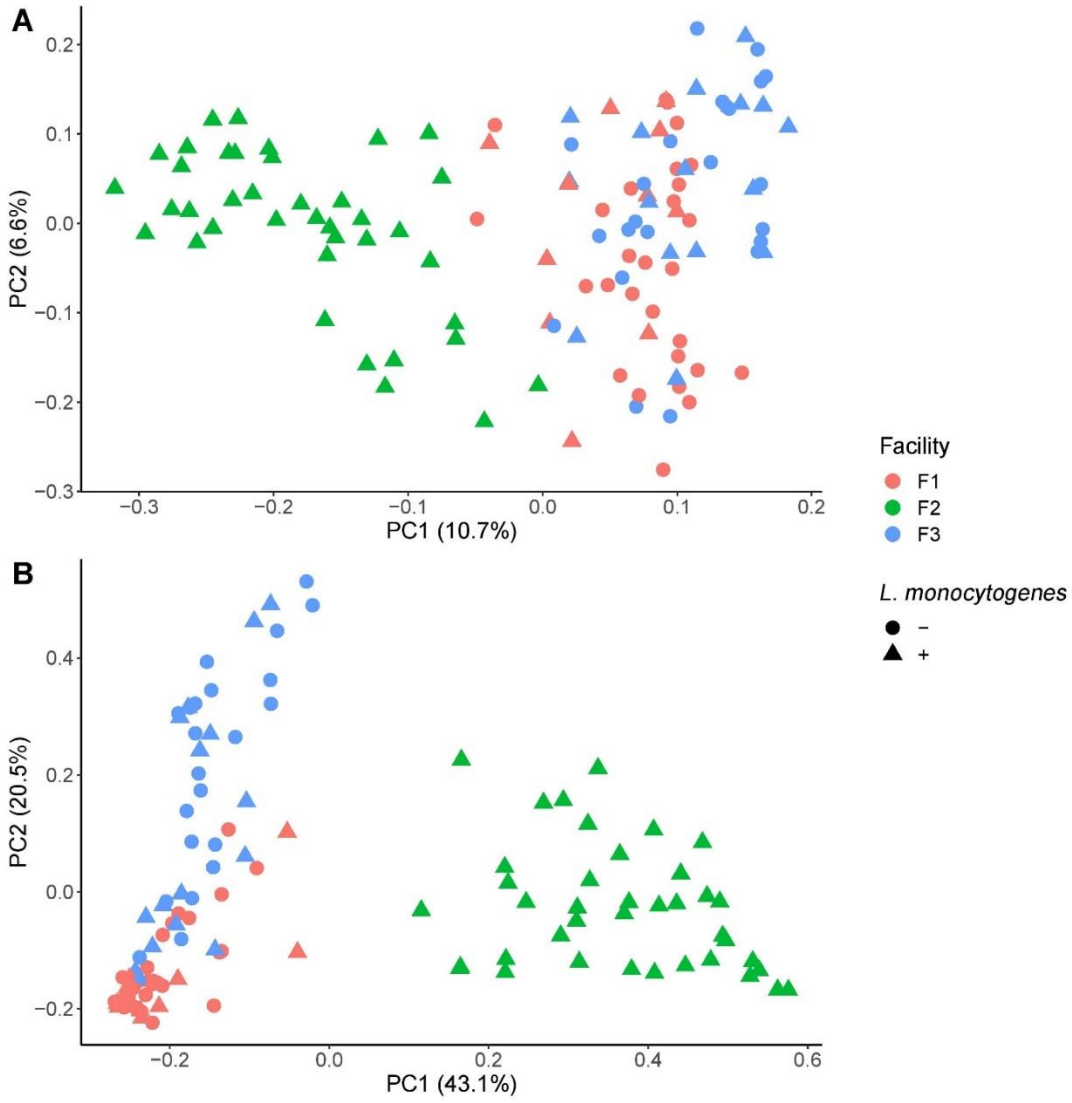

Figure 3. 5**:** Clustering of bacterial (A) and fungal (B) communities based on the UniFrac distances calculated using 16S rRNA V4 gene sequences for bacteria and ITS2 sequences for fungi, respectively. The colors red, green and blue indicate samples from facilities F1, F2 and F3, respectively. The circles and triangles indicate samples positive (+) or negative (-) for *L. monocytogenes* culture, respectively.

3.4.5 Pairwise PERMANOVA analysis indicates that significant differences in microbiota composition exist among facilities.

Beta diversity plot indicated distinct clustering of microbiota among facilities. To statistically evaluate these potential differences, we compared microbiota compositions by the month of sample collection, sampling section, and the individual facility using pairwise PERMANOVA. Our results indicate that the composition of the microbiota did not vary significantly by month ($p > 0.05$, Additional file 2, Table S4). However, we found that different facilities and sections have a significant effect on the composition of microbiota ($p < 0.003$; Table 3.2). Mycobiota composition was also not significantly different in different months (Additional file 2: Table S5), while it was significantly different in different facilities (Table 3.2). Moreover, when comparing samples from different processing sections, mycobiota in washing and fan-drying, as well as in fan-drying and waxing sections was not significantly different ($p = 0.057$ and 1.00, respectively). However, mycobiota differs significantly between washing and waxing sections ($p = 0.012$).

Table 3. 2**:** Comparison of microbiota and mycobiome composition between different facilities

Facility pair	DF[a]	Sum of squares	F model	R2[b]	P value	P adjusted	Sig[c]
Microbiota							
F1 vs F2	1	2.788379	7.663642	0.091601	0.001	0.003	A
F1 vs F3	1	1.52451	3.90379	0.048856	0.001	0.003	B
F2 vs F3	1	3.258745	8.983004	0.105704	0.001	0.003	C
Mycobiomes							
F1 vs F2	1	10.11127	82.07152	0.519205	0.001	0.003	A
F1 vs F3	1	3.247097	23.38158	0.235271	0.001	0.003	B
F2 vs F3	1	8.281348	65.7637	0.463897	0.001	0.003	C

[a]DF, degree of freedom.

[b]R2, R square.

[c]Significance, letter indicates significant difference between facility pairs.

3.4.6 Communities of environmental samples from facility F2 are predominated by Pseudomonadaceae and Dipodascaceae.

To explain the differential clustering of microbiota from samples collected in facility F2, we examined the taxonomic composition of the samples from different facilities (Figure 3.6). Facilities F1, F2 and F3 contained 12, 8, and 14 unique bacterial families that were present at 10% or higher relative abundance (RA), respectively (Figure 3.6). Families present in less than 10% abundance were grouped in a category “Other”. Top three most abundant families found in facility F1 were Flavobacteriaceae (19.68%), Moraxellaceae (11.93%), and Weeksellaceae (10.27%). The high abundance of Pseudomonadaceae was found in facility F2 (41.97%), where Flavobacteriaceae (18.27%) and Xanthomonadaceae (7.69%) were also relatively highly abundant. Facility

F3 had highly abundant Weeksellaceae (13.22%), Flavobacteriaceae (11.45%) and both Burkholderiaceae and Moraxellaceae present at 8.67%. Pseudomonadaceae was identified as a predominant family in samples from facility F2 (41.97% RA), which also had the highest occurrence of *L. monocytogenes*. Samples from facility F2 had significantly higher RA of Pseudomonadaceae compared to Facilities F1 and F3 which contained 7.76% and 6.48% Pseudomonadaceae respectively ($p < 0.0001$). There was no significant difference in Pseudomonadaceae abundance between facilities F1 and F3 ($p = 0.844$). Another bacterial family that was frequently identified in high abundance in facility F2 was Flavobacteriaceae, however, it was frequently present in high RA also in samples from facilities F1 and F3.

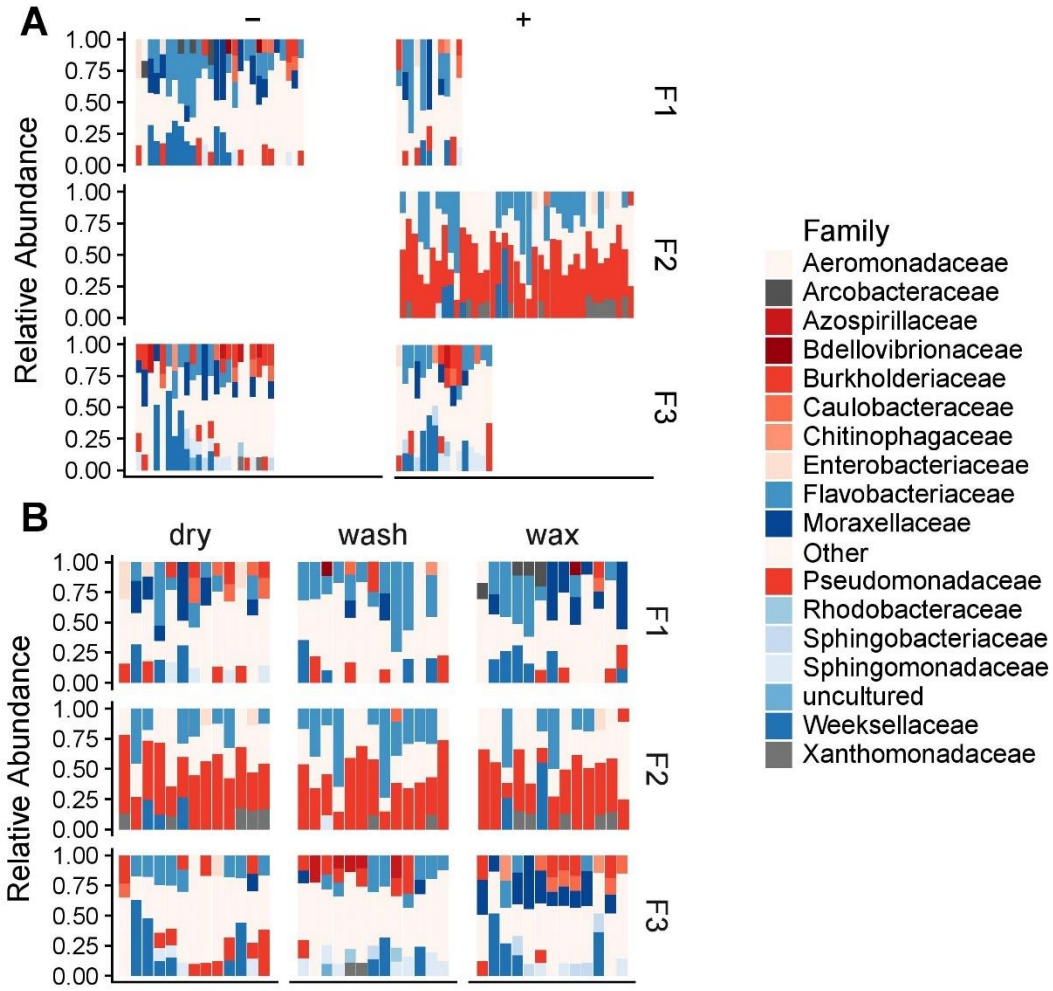

Figure 3. 6**:** Relative abundances of bacterial families in samples collected in three apple packing facilities, shown by the presence (+) or absence (-) of *L. monocytogenes* culture in facilities F1, F2 and F3 (A) and in three sections (washing, fan drying and waxing) within facilities (B). Families with relative abundance less than 10% are shown under the category "Other". Each bar represents an individual sample.

We further explored the difference in fungal communities among three monitored facilities by comparing mycobiota taxonomy at a family level (Figure 3.7). The top three abundant families found in facility F1 were Aureobasidiaceae (30.04%), Aspergillaceae

(12.69%) and Bulleribasidiaceae (9.50%). Facility F2 had three highly abundant families, including Dipodascaceae (56.08%), Trichosporonaceae (10.88%) and Aspergillaceae (4.52%), and the most abundant three fungal families found in facility F3 included Trichosporonaceae (30.63%), Aureobasidiaceae (18.2%) and Pleosporaceae (9.9%). A higher abundance of Dipodascaceae was observed in facility F2 whereas it was present at very low RA in both facilities F1 (0.93%) and F3 (0.48%) ($p < 0.01$). In contrast, Aureobasidiaceae was present at a relatively high level in facilities F1 and F3 and was rarely found in environmental samples of F2.

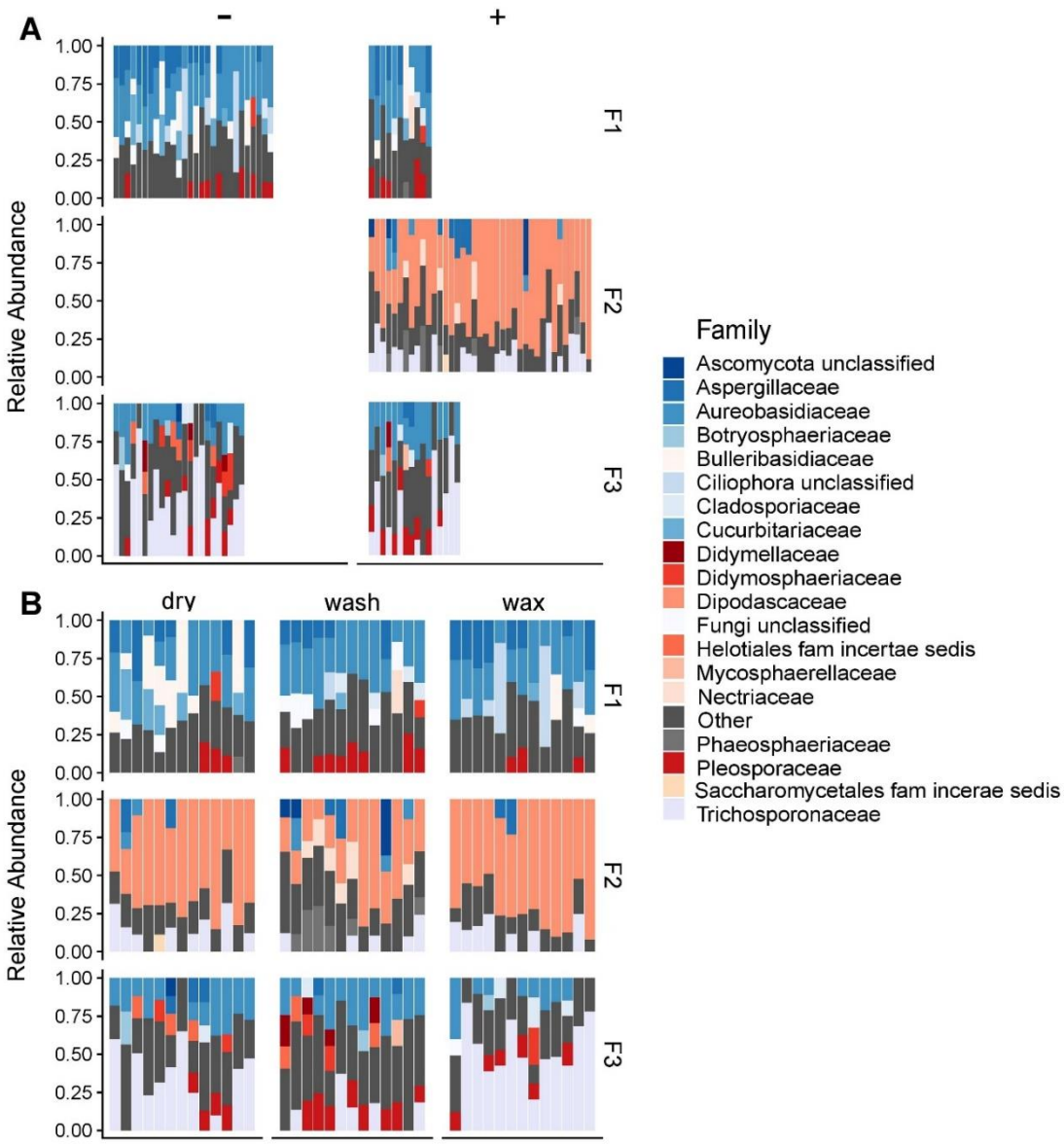

Figure 3. 7**:** Relative abundances of fungal families in samples collected in three apple packing facilities, shown by the presence (+) or absence (-) of *L. monocytogenes* culture in facilities F1, F2 and F3 (A) and in three sections (washing, fan drying and waxing) within facilities (B). Families with relative abundance less than 10 % are shown under the category "Other". Each bar represents an individual sample

3.4.7 Network analysis of microbiota showed co-occurrence patterns between Pseudomonadaceae, Enterobacteriaceae and Rhizobiaceae.

In order to further explore the relationships between different microbial taxa, especially those that were highly abundant in F2, network analysis was carried out to investigate the co-occurrence or co-exclusionary relationship of microbiota in apple packing house built environment. Since facility F2 was previously identified as having the highest occurrence of *L. monocytogenes*, as well as having a relatively higher abundance of bacterial family Pseudomonadaceae, we sub-grouped the network to assess the interactions with a focus on the microbial families in direct relationship with Pseudomonadaceae. Families that co-occurred or co-excluded with Pseudomonadaceae were analyzed with all samples combined as well as with just samples from individual facilities. Both analyses were carried out to minimize potentially spurious relationships induced due to the significantly different composition of microbiota found among facilities. In facility F1, Pseudomonadaceae co-occurred only with Enterobacteriaceae, and Enterobacteriaceae was positively correlated with the occurrence of Rhizobiaceae (Figure 3.8A). No co-occurrence or co-exclusion relationships between Pseudomonadaceae and other taxa have been found when samples from facility F2 were analyzed separately from other samples. Families Rhizobiaceae, Arcobacteraceae, Paludibacteraceae, and one Parcubacteria unclassified family were found to co-occur with Pseudomonadaceae in samples from facility F3 (Figure 3.8B). A similar trend of the relationship was observed when samples from all facilities were analyzed together. In the network of all analyzed samples, Pseudomonadaceae was shown to have a significantly

positive correlation with families Rhizobiaceae, Enterobacteriaceae, and Dysgonomonadace. On the other hand, Pseudomonadaceae had co-exclusionary relation with Spirosomaceae and Bdellovibrionaceae (Figure 3.8C).

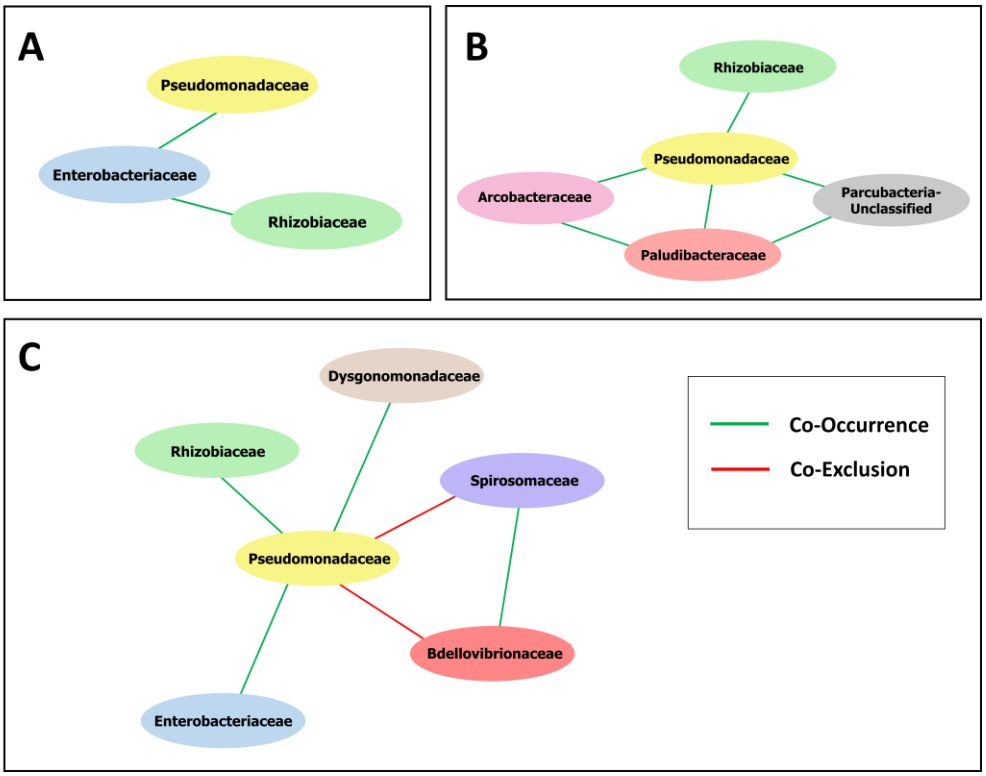

Figure 3. 8**:** Microbial networks showing co-occurrence of microbial families in samples collected from three facilities F1, F2 and F3. Green edges represent positive relationship (i.e., co-occurrence) among families, whereas the red edges represent negative relationship (i.e., co-exclusion) between two connected nodes. Interaction network was sub-grouped with focus on Pseudomonadaceae, a bacterial family that predominated in microbiota of facility F2 that had the highest occurrence of *L. monocytogenes*. Interaction correlation between Pseudomonadaceae when only facility F1 samples were analyzed is shown in a panel (A). Interaction correlation between Pseudomonadaceae when only facility F3 samples were analyzed is shown in a panel (B). Interaction correlation between Pseudomonadaceae and other families when all samples are analyzed together is shown in a panel (C).

3.4.8 PICRUSt predicted the differential abundance of bacterial taxa associated with certain functional categories in microbiota found in facility F2 compared to those found in facilities F1 and F3.

PICRUSt analysis was performed for functional profile predictions of microbiota based on 16S rRNA marker gene. Firstly, the absolute KO abundance in tier 1 functional categories were plotted for samples grouped by individual facility (Figure 3.9A). Facility 2 microbiota samples showed a substantially higher abundance of identified KO in every category, compared to microbiota found in samples from facilities F1 and F3. We further calculated the relative abundance of tier 1 categories of the total absolute abundance in each facility (Figure 3.9B). Since categories *metabolism*, *environmental information processing* and *cellular processes* were considered to be more relevant to our environmental samples, we plotted each of these three categories separately. Microbiota showed a significant difference in functional category *metabolism* when F2 and F1 ($p = 0.0016$) as well as F2 and F3 (8.1×10^{-8}) were compared. There was no significant difference detected in the relative abundance of *metabolism* between F1 and F3 ($p = 0.0522$) (Figure 3.9C). Comparing the microbiota *environmental information processing* profile, facility F2 again showed a significantly higher relative abundance compared to F1 ($p = 0.0194$) and F3 ($p = 0.0019$). When *cellular processes* profile was compared in microbiota from different facilities, facility 2 KO abundance in this category was significantly different from that in F1 ($p = 0.0279$) and F3 ($p = 0.0016$). The abundance of cellular processes KO in facilities F1 and F3 was not significantly different ($p =1.000$).

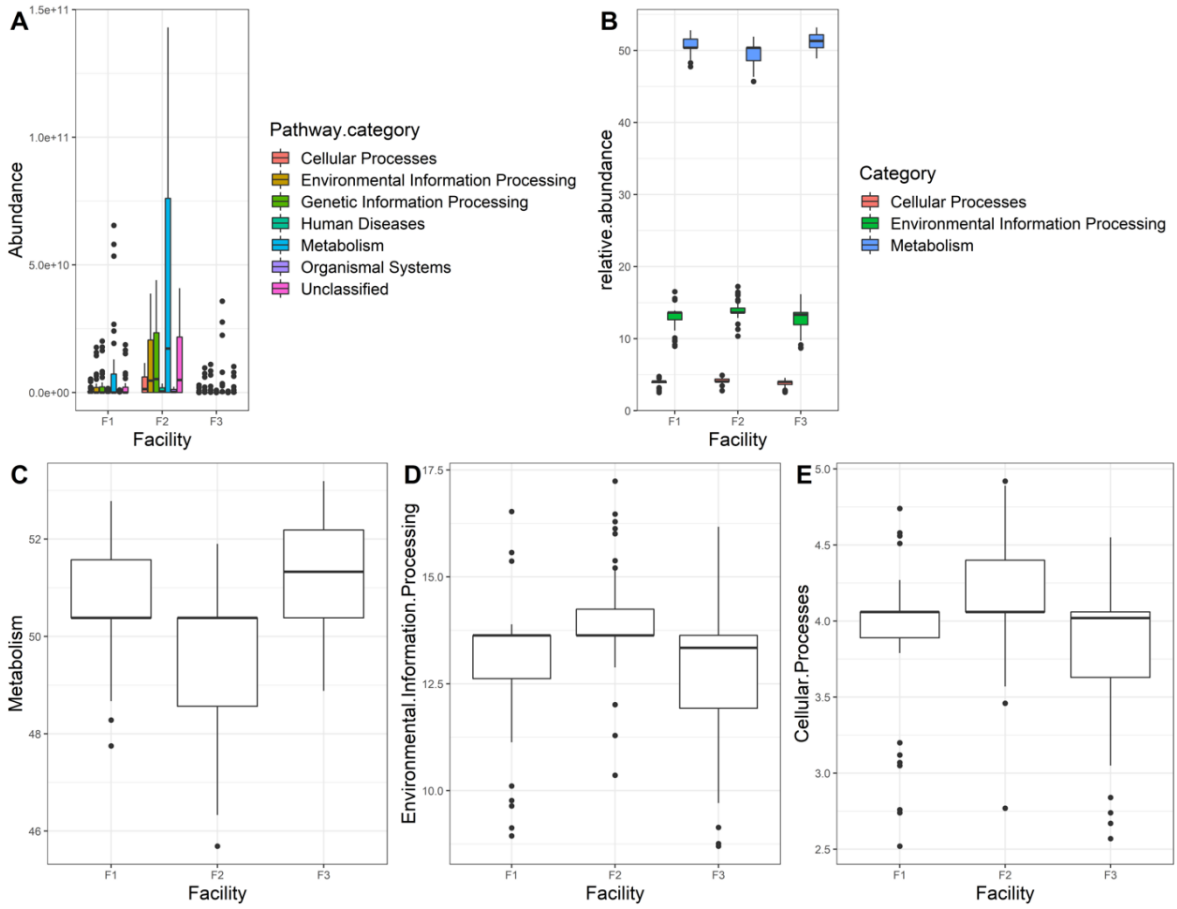

Figure 3. 9**:** Predicted functional profiles of microbiota in facilities F1, F2 and F3 derived from PICRUSt inference based on tier 1 KEGG ortholog categories (KO). Absolute abundance of tier 1 functional categories by facility is shown in a panel (A). Relative abundance of selected tier 1 functional categories by facility is shown in a panel (B). Relative abundance of predicted metabolism of microbiota by facility is shown in a panel (C). Relative abundance of "Environmental information processing" functional category in microbiota from three facilities is shown in a panel (D). Relative abundance of "Cellular processes" functional category in microbiota from three facilities is shown in a panel (E).

3.5 Discussion

3.5.1 Higher occurrence of *L. monocytogenes* was observed in facility F2.

Facility F2 had a significantly higher occurrence of *L. monocytogenes* compared to Facilities F1 and F3. This may be explained by a different facility equipment set-up. For example, while both F1 and F3 were fully equipped, facility F2 did not install a dripping pan under the wet processing line to contain water and organic residues, including leaves, stems and apple pieces during processing. Water and organic residues therefore accumulated on the concrete floor underneath the conveyor where they potentially created a nutrient-rich environment suitable for microbial growth. The lack of a proper drainage system and poor cleaning and sanitizing practices may also have contributed to the unique microbiota in the built environment of F2. The presence of cracks in the floor and between the equipment-floor junctions adds on the difficulties of cleaning specific niches, and it may promote the growth of microbial biofilm and persistence of *L. monocytogenes* contamination in these areas (Ferreira et al., 2014).

3.5.2 Reduced diversity of microbial communities in facility F2 is indicative of the persistence of *L. monocytogenes.*

Rarefaction curves indicated the lowest bacterial and fungal species richness in environmental samples from facility F2, while microbial richness was substantially higher in facilities F1 and F3. The microbiota and mycobiota rarefaction curves for samples collected in facilities F1 and F3 did not reach a plateau, indicating a considerable undiscovered diversity at a used sequencing depth. The indications of rarefaction curves were confirmed by low alpha diversity in samples from facility F2. Lower diversity in microbial species may result in less competition for nutrients among microorganisms as

well as fewer inhibitory secondary metabolites produced by certain microorganisms (Giaouris et al., 2014). Leriche and Carpentier found that a decreased adhesion was found for *L. monocytogenes* when it was co-cultured with *Staphylococcus sciuri* due to competition for nutrients (Leriche & Carpentier, 2000). Secondary metabolites, including bacteriocin-like compounds produced by isolated lactic acid bacteria, have been shown to have antagonistic activity against *L. innocua*, a close relative which is commonly used to model the behavior of *L. monocytogenes* (Ammor, Tauveron, Dufour, & Chevallier, 2006). Production of enzymes that are lytic to cell wall is another mode of antimicrobial action found in yeast, including *Candida guilliermondii* and *Candida oleophila* that have been shown to secrete hydrolytic enzymes that can be used to control *Botrytis cinerea* on tomato plants (Saligkarias, Gravanis, & Epton, 2002). Lower alpha diversity in F2, as well as distinct clustering of microbiota from F2 based on the beta diversity, indicate the ecologically distinct character of facility F2 that potentially supports the growth and/or persistence of *L. monocytogenes.* Based on our collected data it is not possible to speculate whether the lower microbial diversity in facility F2 led to the persistence of *L. monocytogenes*, or the establishment of *L. monocytogenes* led to reduced alpha diversity and distinct microbial ecology compared to facilities F1 and F3. It is noteworthy, though, that facilities F1 and F3 both have a similar microbiota composition despite different locations.

3.5.3 Pseudomonadaceae and Dipodascaceae predominate in facility F2 and represent microorganisms that are known biofilm formers and indicators of unhygienic conditions, respectively.

Pseudomonadaceae was predominant in the environment of the facility F2 with persistent *L. monocytogenes* contamination and is comprised of known biofilm formers, including *Azomonas*, *Azorhizophilus*, *Azotobacter*, *Mesophilobacter*, and *Pseudomonas* (Drobish et al., 2016). *Pseudomonas* species are known for their ability to adapt and thrive in the processing environment (Rossi et al., 2018). They may have a growth advantage in a cold and humid food processing environment that allows them to outcompete a number of other microorganisms because they can grow on a variety of nutrient sources, tolerate low-temperatures and form a biofilm (Sterniša, Klančnik, & Možina, 2019). These properties make *Pseudomonas* successful in the colonization of environmental surfaces. Previous research has demonstrated that *L. monocytogenes* adheres to surfaces substantially better when co-cultured with *P. fragi*. Biofilm-forming *Pseudomonas* could shelter *L. monocytogenes* and enhance its survivability under recurrent cleaning and sanitizing treatments (Puga et al., 2018). This protection is conferred by an EPS that provides a physical barrier that reduces diffusion of chemicals into the core of the biofilm structure, as well as provides nutrients for bacterial growth. *L. monocytogenes* can survive for a longer period of time when co-cultured with *P. pudida* even with no added nutrients (Hassan et al., 2004). These previous findings and our observation of significantly higher relative abundance of Pseudomonadaceae in facility F2 led us to hypothesize that biofilm formation may support *L. monocytogenes* persistence in this facility. Further studies investigating the mechanisms of microbial interactions in microbiota in F2 are warranted.

Another, fungal, family Dipodascaceae was the predominant family detected in facility F2 mycobiota, whereas it was low-abundant or absent in facilities F1 and F3. Dipodascaceae are white filamentous fungi belonging to the order of Saccharomycetales. Microorganisms belonging to this family have been previously shown to have an ability to elevate granular sludge lipid accumulation due to the production of linoleic acid (M. Li, Li, Sun, Jiang, & Chen, 2016). For example, species *Geotrichum candidum*, referred to as "machinery mould", is a common species belonging to Dipodascaceae family and is ubiquitously present in the natural environment (Thornton, Slaughter, & Davis, 2010). It is a plant pathogen that causes post-harvest sour-rot spoilage of fruit and vegetable during storage (Thornton et al., 2010). *G. candidum* can form a white slime on all surfaces that have contact with produce residues and can be readily removed with good sanitation practices (Pitt & Hocking, 2009). Therefore, it has been historically used as a fungal indicator of poor hygienic status in fruit and vegetable processing plants that are associated with inadequate sanitation (National Research Council (US) Subcommittee on Microbiological Criteria, 1985). Although there is no direct information indicating that Dipodascaceae could promote the survival of *L. monocytogenes*, the presence of *G. candidum* does indicate the overall poor hygienic status of facility F2. With reduced hygienic status, the risk of foodborne pathogen contamination and colonization in the facility increases (Muhterem-Uyar et al., 2015).

The unhygienic environmental conditions in facility F2 may have shaped the microbial communities in the environment toward a predominance of Pseudomonadaceae and Dipodascaceae families, as well as *L. monocytogenes.* Furthermore, inadequate

cleaning and sanitizing of the facility presents an increased risk for cross-contamination of produce from the produce processing environment.

Although *L. monocytogenes* is not able to grow on apples, the pathogen can survive on the surface of the fresh fruit (Smith, Moorhouse, Monaghan, Taylor, & Singleton, 2018). Given that apples are consumed raw, their contamination with *L. monocytogenes* presents a health risk for certain groups of consumers, such as elderly and immunocompromised that can get sick by ingestion of as few as 0.1 million CFU of *L. monocytogenes* (Bortolussi, 2008). Additionally, based on two recent outbreaks in the United States and Europe, which involved immunocompromised patients and possibly hypervirulent strains, the probability of infection after consumption of one cell of *L. monocytogenes* was estimated to be almost 100,000 times higher than that estimated by FAO/WHO in 2004 based on epidemiologic data of patients from all susceptible population groups (Pouillot et al., 2016).

In cases when fresh apples are coated with caramel and punctured with a wooden stick, the risk increases, as this creates microenvironments suitable for pathogen growth (Glass et al., 2015). In order to better understand the role and effects of cleaning and sanitizing procedures on the persistence of Pseudomonadaceae, Dipodascaceae and *L. monocytogenes*, as well as the effect of the *Pseudomonas* and Dipodascaceae on *L. monocytogenes* survival, growth and/or persistence of *L. monocytogenes* in natural biofilms is needed. Better understanding of these microbial interactions would allow for precise optimization of cleaning and sanitizing procedures to effectively reduce *L. monocytogenes* in built environments of apple packinghouses.

3.5.4 Co-occurrence and co-exclusion relationships between Pseudomonadaceae and other bacterial families.

We applied network analysis to describe the co-occurrence and co-exclusion relationship between Pseudomonadaceae and other bacterial families that may contribute to *L. monocytogenes* survival and persistence in apple packing house environments. The co-occurrence of Enterobacteriaceae, *Pseudomonas*, and Rhizobiaceae in the environment of the facility 2 is noteworthy, as they are related due to the ease of genetic material exchange (Sanderson, 1976). For example, an antibiotic-resistant R factor is inter-transferable among genera comprising Enterobacteriaceae, *Pseudomonas* and Rhizobiaceae (Datta, Hedges, Shaw, Sykes, & Richmond, 1971). Another study based on Adansonian analysis has shown that members of Rhizobiaceae are highly related to strains in *Escherichia coli* and *Enterobacter aerogenes*, while genera *Rhizobium* and *Phytomyxa* from the family of Rhizobiaceae are closely related to members in Pseudomonadaceae family (Moffett & Colwell, 1968). Besides genetic relatedness, mutualism based on metabolites has also been found between *Pseudomonas aeruginosa* PA14 and *Enterobacter aerogenes*, where the co-culture of the two organisms had at least 14 fold increase in current density compared to either species alone (Venkataraman, Rosenbaum, Perkins, Werner, & Angenent, 2011). In contrast, Pseudomonadaceae and Bdellovibrionaceae were found to co-exclude each other when all samples were analyzed together. *Bdellovibrio*, a genus from the family of Bdellovibrionaceae that was shown to negatively co-occur with Pseudomonadaceae in our study, has been previously been shown to have predatory activity toward Gram-negative bacteria, especially *Escherichia*

coli and *Pseudomonas* (i.e., *Pseudomonas syringae* (Barel, Sirota, Volpin, & Jurkevitch, 2005) and *Pseudomonas aeruginosa* (Iebba et al., 2014)). Furthermore, *B. bacteriovorus* has been shown to reduce existing *E. coli* and *P. fluorescence* biofilm biomass, and *Bdellovibrio*-treated biofilm has been more easily washed off compared to a control (Kadouri & O'Toole, 2005). *Pseudomonas aeruginosa* is also considered as one of *B. bacteriovorus*' favorite prey (Pantanella et al., 2018). Limited information is available about characteristics of microorganisms belonging to the family Spriosomaceae, another family that was negatively co-occurring with Pseudomonadaceae. Our network analysis predicts the potential mechanism underlying the high prevalence of certain bacterial families in the processing environments. The positive relationship may indicate the mutualistic interactions whereas the negative correlation may suggest the inhibitory interactions among microbiota.

3.5.5 Predicted bacterial functional profiles and putative underlying mechanisms.

Samples collected in facility F2 had a substantially higher number of identified KOs compared to samples from F1 and F3, indicating a higher number of functional units were characteristic of microbiota detected in facility F2. This is contradictory to what has been found in other studies where they speculated a decline in functionality in less diverse microbiota communities. However, this may also be a result of urbanization-introduced biotic similarity (Staley et al., 2014), as some of the other studies have shown that low functionality can also be found in highly diverse bacterial communities, as a result of functional redundancy (Shade & Handelsman, 2012).

Facility F2 microbiota had a higher relative abundance of KO in the *metabolism* and *cellular processes* category, whereas it had lower relative KO in the *environmental information processing* category compared to the microbiota from facilities F1 and F3. This is consistent with our hypothesis that led to further analyses of sub-tier categories. Category *metabolism* includes the production of secondary metabolites, which we proposed earlier as one of the potential mechanisms through which persistent colonization of *L. monocytogenes* is inhibited in facilities F1 and F3. On the other hand, the bacterial secretion system which belongs to tier 1 category *environmental information processing*, plays a crucial role in the *P. aeruginosa* biofilm formation (KEGG). For instance, previous studies have shown that Type II (Cianciotto & White, 2017) secretion system is involved in biofilm formation, and Type VI secretion Systems (T6SSs) (Gallique, Bouteiller, & Merieau, 2017; Gallique, Decoin, et al., 2017) is also play a role in the biofilm formation in gram-negative bacteria including *Pseudomonas* spp.. Cell communication, as one of the sub-categories of *cellular processes*, may also help explain the presence of the biofilm-formers in facility F2 since quorum sensing and bacterial biofilm formation are highly linked (Nadell, Xavier, Levin, & Foster, 2008). Although a clear association between the diversity and functionality of native microorganisms present in the built apple processing facility environment needs to be further investigated to validate these functional predictions, our results provide foundational information that may be used to develop targeted study designs to help elucidate the impact of built environment microbial communities on the survival, growth and persistence of pathogenic microorganisms such as *L. monocytogenes* in food processing facilities. This

is particularly important as the use of new biocontrol products is gaining in popularity and needs to be carefully assessed prior to application to avoid adverse outcomes.

3.6 Outlook, novelty and importance.

When designing the cleaning and sanitizing protocols, it is important to consider the microbial landscape in the built environment of food processing facilities. It has been shown that planktonic *L. monocytogenes* could be reduced by 5 logs within 30 s of treatment, whereas the only weak effect was observed in attached cells even after an extended treatment time (Campana, Ciandrini, & Baffone, 2018). The complexity of in-house microbiota community is likely to reduce the effectiveness of disinfectants, and bacteria such as Pseudomonadaceae may help foodborne pathogen such as *L. monocytogenes* in survival and persistence in food processing environments and thus increase the food safety and public health risk. Our longitudinal survey of apple packinghouse environmental microbiota suggests that the composition and diversity of microorganism found in food processing environments is associated with persisting *L. monocytogenes* contamination. Our study provides a data baseline needed for further in-depth investigation of microbial interactions between non-pathogenic and pathogenic microorganisms found in food processing environments. Further research in the area may lead to the optimization of pathogen control strategies and the development of novel biocontrol methods to complement physical and chemical interventions and improve food safety.

3.7 Acknowledgements

This work is partly supported by the U.S. Food and Drug Administration grant number 1U18FD006222-01 to Edward G. Dudley for support of GenomeTrakr in Pennsylvania.

Chapter 4

Conclusion and Future directions

4.1 Research conclusions

In the present study, the composition and diversity of microbiota and its potential association with *L. monocytogenes* occurrence was investigated. Zone 3 floor samples underneath the wet processing sections were collected in three apple packing facilities over a 6-month period. The first objective of this project was to determine the occurrence of *L. monocytogenes* in the processing environment through FDA BAM enrichment method. The occurrence of *L. monocytogenes* in three facilities was 28%, 100%, and 41% in facility F1, F2, and F3, respectively. Facility F2 had significantly higher *L. monocytogenes* occurrence compared to F1 and F3. The high *L. monocytogenes* occurrence level in F2 suggests there are some factors that may contribute to the persistence of *L. monocytogenes* in this particular facility.

The second objective of this project was to determine the potential association between diversity of background microbiota and the occurrence of *L. monocytogenes* in the three apple packing houses. Amplicon sequencing and downstream bioinformatic

analysis revealed a different pattern of microbiota and mycobiota in facility with high *L. monocytogenes* occurrence level compared to those in facilities with lower *L. monocytogenes* occurrence level. Compared to F1 and F3 that had lower *L. monocytogenes* occurrence level, facility F2 with high *L. monocytogenes* occurrence level had relatively lower microbial species richness and evenness as shown by alpha diversity indices. F2 microbiota also showed distinct bacterial and fungal clustering compared to the other two facilities due to microbial compositional difference.

After further investigation of the compositional differences, we found that bacterial family Pseudomonadaceae and fungal family Dipodascaseae predominated in F2 environment, whereas these two families presented only a minor proportion of the microbial communities found in F1 and F3 processing environment ($P < 0.05$). Members in Pseudomonadaceae family are known for their ability to form biofilm that protect bacteria communities inside this matrix from cleaning and sanitizing procedures, which may explain the persistence of *L. monocytogenes* observed in F2. *Geotrichum*, belongs to fungal family Dipodascaseae, has been historically used as an indicator for poor sanitation. The high relative abundance of this fungal family in F2 further suggests the insufficient sanitation procedures failed to remove *L. monocytogenes* from the processing environment at the first place, and can promote the growth and persistence of foodborne pathogens in the processing environment.

Co-occurrence relationship has been found among bacterial family Pseudomonadaceae, Enterobacteriaceae, and Rhizobacteriacea and co-exclusion relationship has been found between Pseudomonadaceae and Bdellovibronaceae. The

positive and negative association of occurrence between bacterial families reflect interactions among microbiota, and the bacterial families that are negatively associated with biofilm former Pseudomonadaceae can be investigated for biocontrol method development.

Additionally, the inferred microbial functional profile showed functional category "environmental information processing" and "cellular processes" were at relatively higher abundance in facility F2 compared to F1 and F3, whereas functional category "metabolism" was found at lower level in F2 microbiota compared to F1 and F3. Bacterial secretion system and cell communication are in the sub-tiers of "environmental information processing" and "cellular processes", respectively, and they both played a crucial role in biofilm formation. High abundance of these functional categories in F2 helped to affirm that the high *L. monocytogenes* occurrence level was largely attributed to biofilm. Category "metabolism" contains sub-tier category "secondary metabolites production", and the relatively higher abundance of this category in F1 and F3 may potentially explained inhibition of colonization of *L. monocytogenes* in these two facilities. However, the results from PICRUSt inference can only be used as a prediction, further investigation in this is needed to show the actual function profiles for microbiota and their association between the different occurrence of *L. monocytogenes* in processing environments.

Based on all the results, this study provides us with a better understanding of microbiota composition in apple packing facilities and their potential interactions between foodborne pathogen such as *L. monocytogenes*. This information could be used

for optimization of sanitation practices in apple industry as well as potential development of biocontrol method.

4.2 Future direction

Future work could include experiments designed to seek understanding of the effects of the complex microbial community on the survival of *L. monocytogenes* after the sanitizer treatment. The efficacy of sanitizers could be tested by comparing the behavior of *L. monocytogenes* in monoculture as well as in this complex biotic system for which we developed a model assay described in the appendix A.

Based on observation, floor samples collected from F2 had much more foam formed after stomaching compared to samples from F1 and F3. The formation of foam might be due to the residual sanitizers lefted on the floor after sanitization practice and the lack of proper drainage system. It might stimulate the formation of *L. monocytogenes* persister cells by exposing to sublethal sanitizer concentration over a long period of time. Thus, it is worthy to test *L. monocytogenes* isolates collected from each facility for their ability to tolerate sanitizer treatment. Moreover, wholegenome sequencing data might allow us to identify the genetic differences among isolates from different facilities.

In addition, we detected the bacterial family Pseudomonadaceae and fungal family Dipodascaceae as uniquely in high relative abundances in the facility with the highest *L. monocytogenes* occurrence level, hence the next step could be testing potential interactions between the presence of these families and growth of *L. monocytogenes* in *in vitro* studies. Studying the underlying mechanism behind the potential symbiosis

interaction between *L. monocytogenes* and these microbial families may provide insight needed for more effective control of *L. monocytogenes* in food processing environments.

Moreover, further optimization of the microtiter-based MPN quantification method described in appendix A would facilitate the study of microbial interactions in a lab-grown microbiome biofilm. An optimized test can also be used for testing of putative biocontrol strains for their ability to inhibit the growth of *L. monocytogenes* in a natural microbiome.

Appendix A

Development of a method for microtiter plate-based MPN quantification of the growth of *L. monocytogenes* and *Pseudomonas* spp. in a natural microbiome biofilm

Abstract

In this thesis, we identified Pseudomonadaceae as a predominant bacterial family found in the apple packing facility with the highest prevalence of *L. monocytogenes.* *Pseudomonas* spp. are known biofilm formers, hence they may play an important role in promoting the survival and persistence of *L. monocytogenes* in the apple processing environment. In this follow-up study, we worked on the development of a microtiter plate-based MPN assay for quantification of the growth of *L. monocytogenes* and *Pseudomonas* spp. in a natural microbiome. Once fully optimized, this method will allow for quantitative measurement of the effects of putative protective (biocontrol) cultures on the growth of *L. monocytogenes* and *Pseudomonas* spp. in natural microbiomes.

Zone 3 floor samples were collected from apple packing facilities and grown in BHI in the microtiter plates for 0, 2, 5 and 7 days. On each of these sampling days, unattached cells were washed away and the attached cells were detached and quantified using an NPM approach with *L. monocytogenes* and *Pseudomonas* spp. selective media.

The preliminary results indicate that both *Pseudomonas* spp. and *L. monocytogenes* grow in a microbiome and attach to the surface through the first two days. However, after day two, levels as MPN/well for both organisms remained

unchanged, likely due to the lack of nutrients, as medium was not replaced throughout the seven-day incubation. The assay will be further optimized based on this finding to allow for quantitative assessment of the efficacy of putative biocontrol isolates.

Background

A biofilm is a collection of bacteria imbedded in an extracellular matrix and irreversibly attached to an abiotic surface (Colagiorgi et al., 2017). The formation of biofilm can be characterized as the attachment to a surface followed by production of extracellular polymeric substances (EPSs), which are natural polymers that consist of proteins, polysaccharides, nucleic acids, lipids produced by cells or other dead cells (da Silva & De Martinis, 2013; Sutherland, 1982). The presence of a biofilm in a food processing facility may increase food safety risks by providing physical protection from the cleaners and sanitizers on pathogens in food processing environments (Chaitiemwong et al., 2014; Flemming & Wingender, 2010).

In Chapter 3 of this thesis, we found that among the three monitored apple packing facilities, the facility with the highest *L. monocytogenes* occurrence was colonized by microbiota that was distinct from the other two facilities that had lower *L. monocytogenes* occurrence level. Further analysis of the microbiota composition identified Pseudomonadaceae as the most abundant bacterial family in the facility with high occurrence of *L. monocytogenes*. Members of Pseudomonadaceae, including *Pseudomonas* spp., are known for their ability to form biofilms on abiotic surfaces (Rossi et al., 2018). We therefore hypothesized that the presence of biofilm formers facilitates persistence of *L. monocytogenes* in the apple packinghouse environment by means of

creating a physical barrier that reduces the effectiveness of the sanitizer diffusion and its contact with the pathogen within the biofilm.

Several studies have shown that current sanitation practices are not always effective (Dutta et al., 2013; Granier et al., 2011; Mereghetti, Quentin, Marquet-Van Der Mee, & Audurier, 2000). Microbial interactions within natural microbiomes, including those that result in a biofilm formation and development of antimicrobial tolerance or resistance may lead to the reduced sanitizer efficacy. It is therefore important to study the interactions between *L. monocytogenes* and microbiota present in the same environments to improve our understanding of the role of the microbiome in survival and persistence of *L. monocytogenes.* Understanding the interactions between Pseudomonadaceae and *L. monocytogenes* in a complex microbiome biofilm structure may provide insights into mechanisms that may be targeted in pathogen control interventions.

In this study, we aimed to develop a method for quantitative assessment of *Pseudomonas* spp. and *L. monocytogenes* growth in natural microbiome samples obtained from apple packing facility environments. The assay may be further optimized and applied to evaluate the efficacy of putative biocontrol cultures to inhibit *L. monocytogenes* growth in microbiome biofilms.

Materials and Methods

Sample collection

Environmental samples were collected from the packing house floor underneath a rolling brush conveyor belt used to transport fruit through the washing, air-drying, and

waxing process. This section was identified in a preliminary study as having the highest *L. monocytogenes* occurrence level within each facility. Microbiome samples were collected using 3M hydrated sponges with 10 mL D/E neutralizing buffer by swabbing a 40 cm by 40 cm area 10 by 10 strokes. Sponge samples were stored on ice in a cooler during transportation to the Department of Food Science lab and were analyzed within 24 hours of collection.

Microtiter-based MPN assay inoculum preparation

Ninety ml of Brain Hear infusion (BHI) broth were added into the sample bag and were stomached at 260 rpm for 7 minutes. The concentration of the total aerobic mesophilic bacteria per sponge was calculated by transferring 1 ml of homogenate to a sterile conical tube containing 9 ml of 1 X PBS buffer. The suspension was mixed and ten-fold serially diluted. A 0.1 ml of 10^{-7}, 10^{-8}, 10^{-9}, and 10^{-10} dilutions were spread-plated onto BHI agar. Inoculated BHI agar plates were incubated at 35 ºC for 24 hours to determine counts.

To prepare an MPN assay inoculum, five ml of the above-described homogenate were transferred to a 50 ml conical tube containing 45 ml BHI broth and vortexed for a few seconds until thoroughly mixed. One ml of this suspension was aliquoted into each well of a 12-well sterile microtiter plate (VWR) as shown in Figure 1. Inoculated microtiter plate was covered with a sterile aluminum foil to minimize liquid evaporation and incubated at 15 ºC for 7 days. The attached microbiome, *L. monocytogenes* and *Pseudomonas* spp. were quantified in triplicates (i.e., A, B, C shown in Fig. 1) on days 0,

2, 5 and 7 as described below. The temperature of 15 ºC was selected to approximate the apple packing house environment temperature.

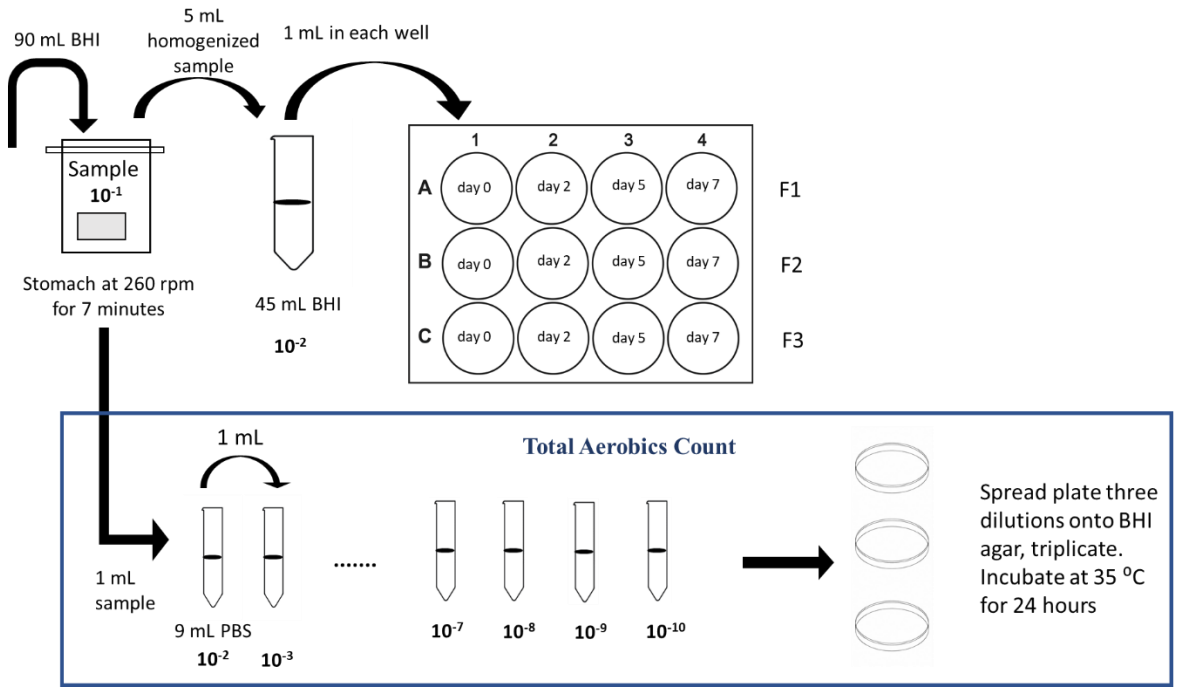

Figure 1A: Microtiter plate-based MPN assay setup and the process of obtaining initial total aerobics counts from environmental samples.

4.3.3 Quantification of attached cells

Attached cells were quantified after incubation in a microtiter plate on days 0, 2, 5 and 7. Medium with planktonic cells was carefully removed using a pipette. Each well was washed two times with 1 ml 1 X PBS buffer to remove non-attached cells. The attached cells were assumed to be incorporated into a biofilm, although the EPS was not confirmed. After washing, 1 ml of fresh 1X PBS and 1 g of sterile 3 mm glass beads were added into each tested well (e.g., wells A1, B1 and C1 on day 0; wells A2, B2 and C2 od day 2). The attached biomass was detached by 2-minute shaking at 1500 rpm at room

temperature using a microplate shaker (Benchmark Scientific Inc. Model H5000-H). Detached biomass was transferred from each well into individual empty 1.7-ml microcentrifuge tubes. Each sample was diluted in a series of seven 10-fold dilutions in 0.9 ml PBS. Dilutions 10^{-4}, 10^{-6}, 10^{-8} (0.1 ml each) were spread-plated onto BHI agar in triplicates as shown in Fig. 2. Inoculated BHI agar plates were incubated at 35 ºC for 24 hours and counted to determine the concentration of total attached aerobic mesophilic microorganisms.

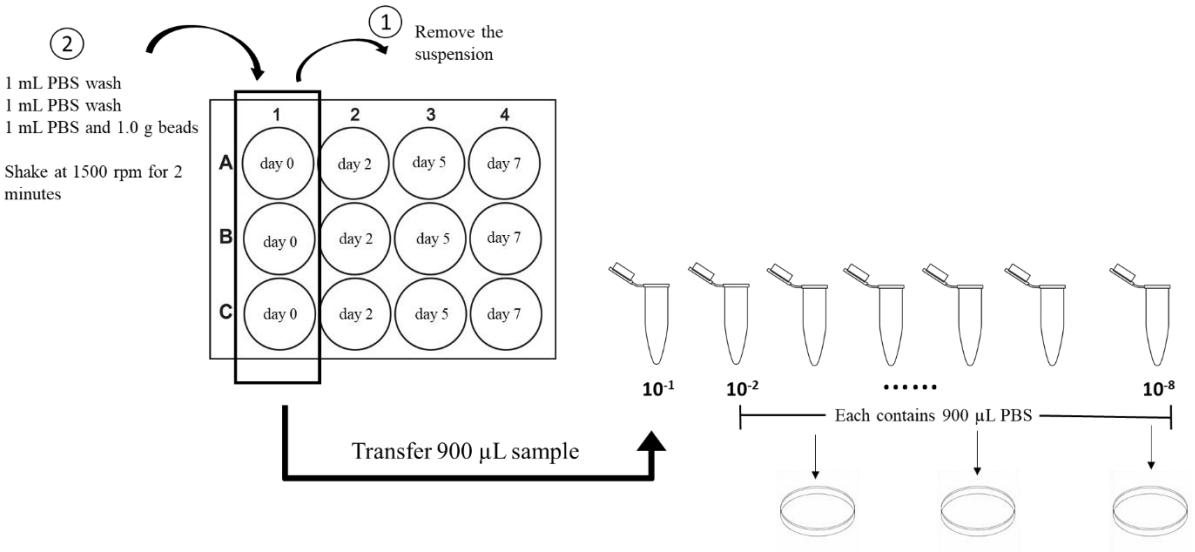

Figure 2A: Illustration of treatment of the microtiter plate with attached biomass and total attached aerobic mesophilic microorganism quantification that were carried out before *L. monocytogenes* and *Pseudomonas* spp. MPN quantification.

MPN quantification of *Pseudomonas* spp. and *L. monocytogenes*

Growth of *L. monocytogenes* (Figure 3A), and *Pseudomonas* spp. (Figure 3B) in a natural attached microbiome was quantified using an MPN assay as described in the following sections.

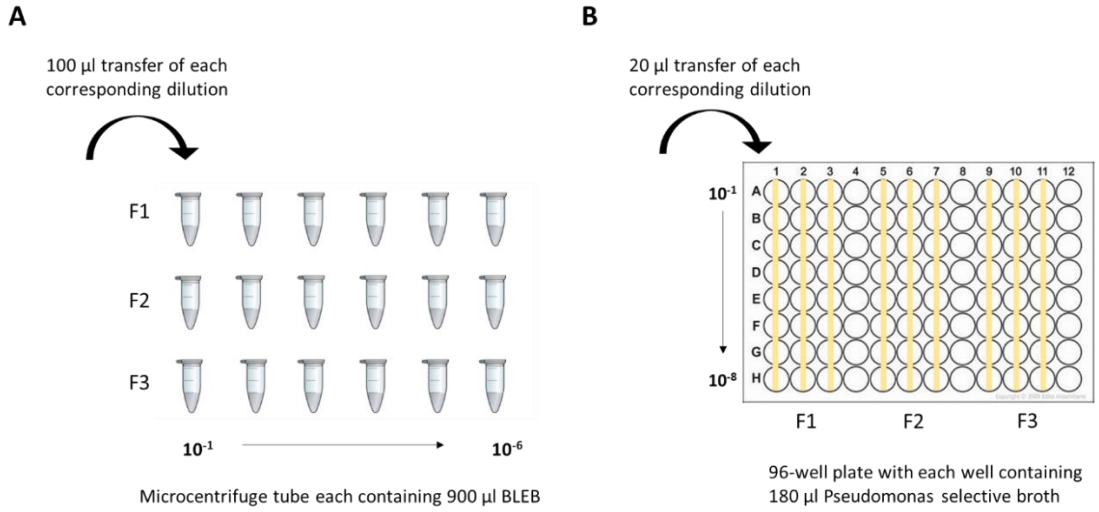

Figure 3A: MPN quantification scheme of *L. monocytogenes* (3A-A) and *Pseudomonas* spp. (3A-B).

Quantification of *L. monocytogenes*

The detached microbiome cells obtained in the process shown in Fig. 2 was used for MPN quantification of *L. monocytogenes.* Three sets of six 1.7 ml microcentrifuge tubes were filled with 900 µl Buffered *Listeria* Enrichment Broth (BLEB, Oxoid) for enrichment of *L. monocytogenes*. Five 10-fold dilutions (10^{-1} to 10^{-6}) prepared for quantification of total attached aerobic mesophilic counts were used in the MPN assay. One hundred µl of each dilution were transferred into tubes containing 900 µl of BLEB and incubated for 4 hours at 30 ºC. After 4 hours, 4 µl BLEB supplement SR0149 (Oxoid) was added and incubation continued for another 40 hours at 30 ºC. After a total 48 hours of incubation, a loopful of the suspension from each well was streaked onto Rapid *L. mono* agar (BioRad Laboratories Inc.). All plates were incubated at 37 ºC for 24

to 48 hours until the colonies began to appear. Colonies appearing blue-green with no background color change were considered as putative *L. monocytogenes*. Since 100 µl out of 1 ml per well were tested to quantify *L. monocytogenes,* the final results were calculated as MPN/0.1well and then expressed in MPN/well.

Quantification and confirmation of *Pseudomonas* spp.

Quantification of *Pseudomonas* was carried out in a 96-well plate instead of microcentrifuge tubes, since the selective medium for *Pseudomonas* does not require addition of very small volumes of selective supplements after the initial incubation and could therefore be conducted in a smaller volume 96-well microtiter plate. This allowed for reduction in media usage and was more suitable for testing larger number of serial dilutions. Twenty µl of each serial dilution (10^{-1} to 10^{-8}) of the detached microbiome suspension prepared as shown in figure 2 were transferred into a 96-well microtiter plate containing 180 µl of *Pseudomonas* selective broth (Millipore) in each well. Each of these dilutions were enriched in a *Pseudomonas* spp. MPN assay. Since 20 µl out of 1 ml per well were tested for the presence of *L. monocytogenes*, we reported the final results as MPN/0.02 well and finally expressed the results as MPN/well. The plate was incubated at 25 ºC for 48 hours. After 48-hour incubation, one loopful of suspension from each well was streaked for isolation onto *Pseudomonas* base agar (Oxoid) with CFC supplement SR0103 (Oxoid). The inoculated plates were incubated at 25 ºC for 24 to 48 hours, until colonies were clearly visible. Colonies appearing brown or fluorescent green were considered as putative *Pseudomonas* spp.

Calculation for MPN

The levels of *L. monocytogenes* and *Pseudomonas* spp. were expressed as MPN/well, and MPN was calculated based on validated spreadsheet calculator provided in FDA BAM: Detection and Enumeration of *Listeria monocytogenes* protocol (FDA, 2017).

Results

Total aerobic bacterial cell counts were at similar level in samples collected from the three facilities.

The initial total aerobic mesophilic counts (TAMC) were determined by plating the serial dilutions of samples stored at 4 ºC within 12 hours of the sampling time. Based on the samples collected during a single sampling visit, facility F1 had the highest total aerobic mesophilic count at 2.27×10^8 CFU/sponge, followed by facility F2 with 2.00×10^7 CFU/sponge and facility F3 with 1.67×10^7 CFU/sponge (Table 1). However, none of the results are based on countable plates (plates had fewer than 30 colonies). Hence these numbers can only be used as estimation of the TAMC.

Total attached cell counts showed an increase and then decrease over the 7-day incubation period

Total attached cell counts (TACC) increased from day 0 to day 2 in samples collected from all three facilities. On day 0, F1 had 5.13×10^5 CFU/ml TACC, which is substantially higher compared to TACC found in F2 (7.33×10^2 CFU/ml) and F3 (1.55×10^4 CFU/ml). TACC in F1 sample increased on day 2 (1.25×10^7 CFU/ml) and day

5 (5.57×10^8 CFU/ml), however, the cell counts were decreased to 6.33×10^7 CFU/ml was observed on day 7. Sample collected from F2 showed consistent increase in TACC, where TACC increased from day 0 (7.33×10^2 CFU/well) to day 2 (5.67×10^6 CFU/well), from day 2 to day 5 (3.43×10^8 CFU/well), and from day 5 to day 7 (5.73×10^8 CFU/well). Sample collected from F3 showed a similar trend as sample from F1 where the TACC were increasing until day 5 (1.55×10^4 CFU/well, 1.00×10^7 CFU/well, and 1.23×10^9CFU/well, respectively), and then declined to 2.07×10^8 CFU/ml on day 7.

L. monocytogenes and _Pseudomonas_ spp. counts showed a similar growth trend as TACC during 7-day incubation

L. monocytogenes was not detected in samples from F1 on day 0, day 2 and day 7, however, a 1.9×10^8 MPN/well of *L. monocytogenes* was detected in the F1 sample on day 5. *L. monocytogenes* MPN counts in the sample F2 increased from 0 MPN/well on day 0, to 2.31×10^4 on day 2, and 9.05×10^5 on day 5. A 1.42×10^4 MPN/well reduction in *L. monocytogenes* was observed on day 7 (8.91×10^5 MPN/well). No *L. monocytogenes* was detected on day 0, however, it was detected at a high level on day 2 (2.55×10^8 MPN/well), day 5 (7.45×10^6 MPN/well) and day 7 (2.22×10^7 MPN/well) in the F3 sample.

Pseudomonas spp. in F1 sample showed a 3-log increase in growth from day 0 (4.66×10^3 MPN/well) to day 2 (7.62×10^7MPN/well). After that, the concentration level decreased from day 5 (1.9×10^8 MPN/well) to day 7 (5.60×10^7 MPN/well). A similar trends have observed in F2 samples where the level of *Pseudomonas* spp. increased from day 0 (7.47×10^3 MPN/well) to day 2 (3.91×10^5 MPN/well) as well as from day 2 to day 5

(1.90×10^8 MPN/well). However, a 1-log reduction was observed from day 5 to day 7 (5.60×10^7 MPN/well). F3 samples showed a more consistent pattern of *Pseudomonas* spp. growth compared to samples from F1 and F2. *Pseudomonas* spp. MPN increased initially, and then declined after day 5. A 3.57 MPN/well, 2.55×10^8 MPN/well, 1.24×10^8 MPN/well, and 1.8×10^6 MPN/well were determined using MPN quantification for day 0, day 2, day 5, and day 7, respectively.

Table 1A: Total aerobic mesophilic counts in samples collected from facilities F1, F2 and F3 on the day of collection.

Facility	Dilutions												Average	TAMC[a] (CFU/ml)
	10^{-7}			10^{-8}			10^{-9}			10^{-10}				
F1	23	16	29	5	4	0	0	0	0	0	0	0	2.27E+01	2.27E+08
F2	0	6	0	0	0	0	0	0	0	0	0	0	2.00E+00[a]	2.00E+07[b]
F3	2	2	1	0	0	0	1	0	0	0	0	0	1.67E+00[a]	1.67E+07[b]

[a] TAMC, total aerobic mesophilic counts.

[b] Calculations were based on non-countable plates to obtain an estimation of the level of total aerobic mesophilic bacteria concentration.

Table 2A: Summary of the total attached cell count, *Pseudomonas* spp., and *L. monocytogenes* MPN on Day 0.

TACC[a]											
Facility	Dilutions									Average	TACC (CFU/well)
	10^{-2}			10^{-3}			10^{-4}				
F1	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	58	55	41	51.33	5.13E+05
F2	9	10	3	0	1	4	1	0	0	7.33[a]	7.33E+02[b]
F3	183	145	138	20	N/A	11	0	0	0	155.33	1.55E+04

	***Pseudomonas* spp.**						***L. monocytogenes***					
	Dilution			logMPN	MPN/0.02 well	MPN/well	Dilution			logMPN	MPN/0.1 well	MPN/well
Facility	10^{-1}	10^{-2}	10^{-3}				10^{-1}	10^{-2}	10^{-3}			
F1	3/3	2/3	0/3	1.97	9.33E+01	4.66E+03	0/3	0/3	0/3	0	0	0
F2	3/3	2/3	1/3	2.174	1.49E+02	7.47E+03	0/3	0/3	0/3	0	0	0
F3	1/3	0/3	0/3	0.553	3.57E+00	1.79E+02	0/3	0/3	0/3	0	0	0

[a] TACC, total attached cell count.

[b] Calculations were based on non-countable plates to get an estimation of TACC

Table 3A: Summary of total attached cell count (TACC), *Pseudomonas* spp., and *L. monocytogenes* MPN on Day 2.

TACC[a]

Facility	Dilutions									Average	TACC (CFU/well)
	10^{-4}			10^{-5}			10^{-6}				
F1	TNTC	TNTC	TNTC	129	115	130	1	0	2	124.67	1.25E+07
F2	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	4	8	5	5.67[b]	5.67E+06[b]
F3	TNTC	TNTC	TNTC	TNTC	TNTC	TNTC	5	11	14	10.00[b]	1.00E+07[b]

***Pseudomonas* spp.**

Facility	Dilutions								logMPN	MPN/0.02 well	MPN/well
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}			
F1	3/3	3/3	3/3	3/3	3/3	3/3	0/3	0/3	6.183	1.52E+06	7.62E+07
F2	3/3	3/3	2/3	3/3	2/3	3/3	1/3	0/3	3.893	7.82E+03	3.91E+05
F3	3/3	3/3	3/3	3/3	3/3	3/3	3/3	1/3	6.708	5.10E+06	2.55E+08

L. monocytogenes

Facility	Dilutions					logMPN	MPN/0.1 well	MPN/well
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}			
F1	0/3	0/3	0/3	0/3	0/3	0	0.00E+00	0.00E+00
F2	3/3	3/3	3/3	0/3	0/3	3.364	2.31E+03	2.31E+04
F3	3/3	3/3	3/3	3/3	3/2	5.027	1.06E+05	1.06E+06

[a] TACC refers to total attached cell count.

[b] Calculations were based on non-countable plates to obtain an estimation of TACC.

Table 4A: Summary of total attached cell count (TACC), *Pseudomonas* spp., and *L. monocytogenes* MPN on Day 5

TACC[a]

Facility	Dilutions									Average	TACC (CFU/well)
	10^{-6}			10^{-8}			10^{-10}				
F1	596	584	492	6	3	9	0	0	0	557.33[b]	5.57E+08[b]
F2	354	315	361	6	4	1	0	0	0	343.33[b]	3.43E+08[b]
F3	612	NA	588	7	11	19	0	0	0	12.33[b]	1.23E+09[b]

***Pseudomonas* spp**.

Facility	Dilutions								logMPN	MPN/0.02 well	MPN/well
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}			
F1	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3	6.579	3.80E+06	1.90E+08
F2	3/3	3/3	3/3	3/3	3/3	3/3	3/3	0/3	6.579	3.80E+06	1.90E+08
F3	3/3	3/3	3/3	3/3	3/3	3/3	2/3	0/3	6.393	2470864.1	1.24E+08

L. monocytogenes

Facility	Dilutions						logMPN	MPN/0.1 well	MPN/well
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}			
F1	2/3	2/3	0/3	0/3	0/3	0/3	1.323	2.10E+01	2.10E+02
F2	3/3	3/3	3/3	3/3	2/3	0/3	4.957	9.05E+04	9.05E+05
F3	3/3	3/3	3/3	3/3	3/3	2/3	5.872	7.45E+05	7.45E+06

[a] TACC refers to total attached cell count.

[b] Calculations were based on non-countable plates to get an estimation of TACC.

Table 5A: Summary of total attached cell count (TACC), *Pseudomonas* spp., and *L. monocytogenes* MPN on Day 7

TACC[a]											
Facility	Dilutions									Average	TACC (CFU/well)
	10^{-7}			10^{-8}			10^{-9}				
F1	2	8	9	0	0	2	0	0	0	6.33[b]	6.33E+07[b]
F2	50	70	52	5	7	NA	0	0	0	57.33	5.73E+08
F3	23	19	20	0	2	2	1	0	0	20.67[b]	2.07E+08[b]

***Pseudomonas* spp.**											
Facility	Dilutions								logMPN	MPN/0.02 well	MPN/well
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}			
F1	3/3	3/3	3/3	3/3	1/3	2/3	0/3	1/3	5.174	1.49E+05	7.47E+06
F2	3/3	3/3	3/3	3/3	3/3	2/3	1/3	0/3	6.049	1.12E+06	5.60E+07
F3	3/3	3/3	3/3	2/3	3/3	1/3	0/3	0/3	4.555	3.59E+04	1.80E+06

L. monocytogenes											
Facility	Dilutions								logMPN	MPN/0.1 well	MPN/well
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}			
F1	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0/3	0	0.00E+00	0.00E+00
F2	3/3	3/3	3/3	3/3	2/3	0/3	0/3	0/3	4.95	8.91E+04	8.91E+05
F3	3/3	3/3	3/3	3/3	3/3	3/3	1/3	0/3	6.346	2.22E+06	2.22E+07

[a]TACC refers to total attached cell count.

[b]Calculation based on non-countable plates to get an estimation of TACC.

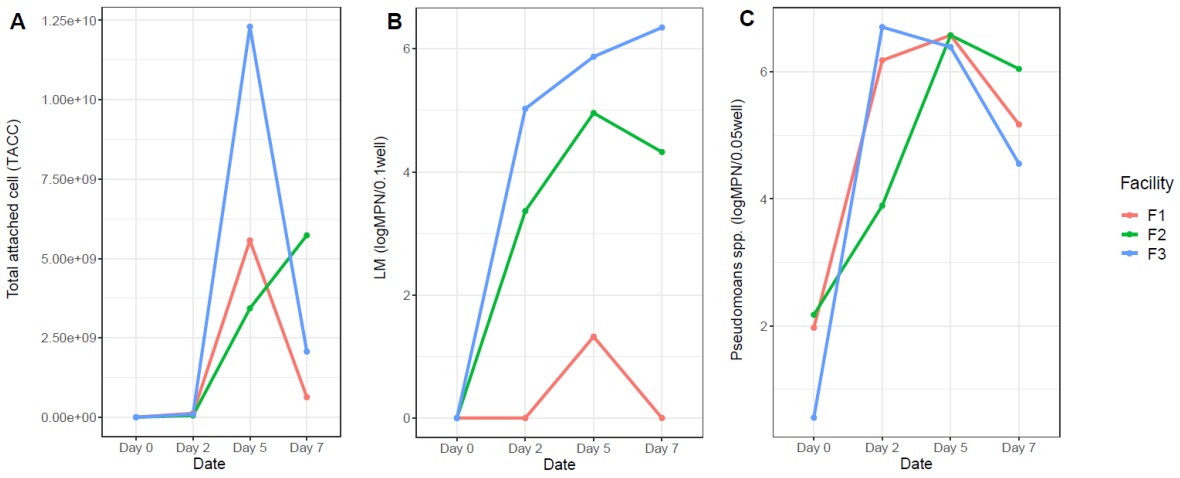

Figure 5A: Quantification of total attached cell counts (TACC) (5A-A), *L. monocytogenes* (LM) (5A-B), and *Pseudomonas* spp. (5A-C) throughout the experiment testing period of seven days.

Discussion

Based on the results obtained from the developed microtiter plate-based MPN quantification method, the goal of monitoring quantities of target microorganisms in an attached microbial community was accomplished. However, the lack of biological and technical replicates necessitates further experiments to verify our results and optimize the protocol to overcome challenges with obtaining high TACC on day 0, which was essentially our time 0.

Unexpected high level of TACC on day 0

We observed an unexpectedly high level of TACC on day 0 for all facilities. This is likely not a valid result, since the homogenate suspension was added into day 0 well and discarded within 5 minutes after deposition. We did not expect any attachment to occur in this short time frame, since most of the leftover unattached cells from suspension

should be removed after the two washes with PBS. Since we did not carry out biological replicates of the experiment, this limits our ability to identify and confirm possible causes of this result. A replication of this experiment needs to be conducted in order to identify a potential error in the procedure.

Growth trends of *L. monocytogenes* and *Pseudomonas* spp. in an attached microbiome were similar to those of the TACC

We observed similar growth when comparing the *L. monocytogenes*, *Pseudomonas* spp. and TACC in that the MPN/well of these microorganisms increased to a relatively high level in the first 2 to 5 days of the incubation period for samples from all facilities. The increase in MPN for attached cells, *L. monocytogenes* and *Pseudomonas* spp. were expected because *Pseudomonas* spp. is known for its ability to attach onto abiotic surfaces and form biofilms, whereas *L. monocytogenes* can grow with in the biofilm matrix (Pang & Yuk, 2019; Puga et al., 2018). Reductions in MPN were observed in multiple samples when day 5 and day 7 samples were compared. One possible cause of this observed reduction was depletion of nutrients in the medium was not replaced at any point after day 0 through day 7. This could have caused the cells to die off due to starvation (Allocati, Masulli, Di Ilio, & De Laurenzi, 2015). It is known that depletion of nutrients can lead to detachment of *Pseudomonas fluorescens* cells from the biofilm, which could explain the reduction in the quantities of attached cells (Allen, Habimana, & Casey, 2018). Starvation has also been shown to be responsible for triggering detachment of *Pseudomonas aeruginosa* incorporated in a biofilm (Hunt, Werner, Huang, Hamilton, & Stewart, 2004). Therefore, if the goal of the experiment is to measure maximum

growth of target microorganisms in an attached microbiome, replacement of the medium should be considered to provide cells with sufficient nutrients to grow. Changing the medium may also better simulate the actual food processing environment where food debris is available for bacterial growth. Furthermore, the removal of planktonic cells during the media replacement may better mimic the real-life situations where the surfaces in the food processing environment are cleaned on daily basis, which would result in reductions of free or loosely attached microbial cells.

MPN quantification of *L. monocytogenes* and *Pseudomonas* spp. indicates the growth of target microorganisms but may be a result of false positive interpretation of the results

Another unexpected result obtained from the experiment is that sample from F3 had substantially higher levels on day 2 of *Pseudomonas* spp. determined by using MPN quantification method compared to TACC determined using the spread plating assay. One explanation is that the MPN quantification was based on quantification of colonies with a typical morphology grown on a selective agar plate, and no molecular confirmation was conducted to identify putative positive colonies. Therefore, this may result in false positive detection when other microorganisms in the environmental sample have the ability to grow on the selective agar and appear similar to *Pseudomonas* spp. Nevertheless, the counts should not exceed the TACC. Another possible explanation of the observed anomaly is that many other bacteria from the sample were injured after the plate was shaken with beads to detach the attached cells. Other studies have reported that injured cells may not be able to form colonies on agar plates (Davis, 2014; Ding et al.,

2017). Therefore, it is possible that the level of total attached cells was underestimated due to inability to delayed growth or inability to inhibited growth. In contrary, the target microorganisms quantified through MPN were given sufficient time to grow first in a broth, albeit selective, and later on a selective plate.

Even though the quantification of *L. monocytogenes* and *Pseudomonas* spp. may be a subject to false positive interpretations, we did observe an increased growth of these two microorganisms over time. Results obtained from samples on all other incubation days suggested that *Pseudomonas* spp. were able to predominate the total attached cell community. Furthermore, *L. monocytogenes* was also shown to be able to attach in this community and increase in population over time.

Optimization is still needed to provide reliable and accurate results; however, the method in its current form did allow us to monitor the growth trend of our target microorganisms in the attached microbiome where the background microbiota is affecting the growth of target microorganisms.

Pitfalls and challenges that were successfully addressed

Optimization of the method based on initial two optimization experiments

Optimization experiments (i.e., trial 1 and trial 2) were conducted to develop and optimize the microtiter plate-based MPN quantification method (results not shown). In trial 1, no *L. monocytogenes* growth was observed throughout the 7-day incubation period. Furthermore, the level of *Pseudomonas* spp. exceeded the upper limit of the BAM MPN quantification, meaning that all sample dilutions tested positive. Therefore, we

proceeded by using 96-well plates for *Pseudomonas* spp. enrichment to be prepare a higher number of dilutions that were needed to obtain a calculable concentration.

In the trial 2, a 96-well microtiter plate was used for *Pseudomonas* spp. enrichment. However, one challenge of the assay was the relatively greater amount of evaporation due to the smaller test volume compared to the microcentrifuge tube format assay. In fact, the broth completely evaporated from wells during the 2-day MPN incubation period. We overcame this challenge by wrapping the 96-well plate with autoclaved aluminum foil, which reduced the evaporation.

Undefined initial cell counts

When using a natural microbiome in biofilm growth experiments, there is no a prior knowledge regarding the initial cell concentration, hence the concentration of the total aerobic mesophilic microorganisms, as well as *L. monocytogenes* and *Pseudomonas* had to be estimated based on observations from previous experiments. We selected dilutions of 10^{-7}, $10^{-8,}$ 10^{-9}, 10^{-10} for spread plating to obtain the initial total aerobic mesophilic microbial counts. However, the concentration of the microorganisms depends heavily on the hygienic condition of each facility. Specifically, whether the facility is in operation on the sampling day, as well as the length of time since the last sanitation procedure. For instance, facility F2 was not in operation during sample collection for attached microbiome growth and the facility was cleaned and sanitized on the day before sampling. We typically collected samples in the middle of the processing shift when increased moisture in the processing environment enhances the movement of pathogens that may be residing in difficult-to-clean niches. Since the trial 1 samples were collected

on a day when the processing line was not operational, the overall expected TACC were lower and the probability of detecting *L. monocytogenes* was expected to be lower as well. To develop a protocol that is robust to these changes and allows for valid quantification of TACC and is not overly labor-intensive, we propose covering a broader range of dilutions in the initial TACC quantification. This can be achieved by spread plating odd dilutions from 10^{-5} to 10^{-11}.

MPN quantification in 96-well microtiter plates

MPN quantification of both *L. monocytogenes* and *Pseudomonas* spp. was initially conducted in 1-ml volumes in 1.7- ml microcentrifuge tubes, however, rapid growth of both target microorganisms in first two days necessitated preparation of additional MPN dilutions, resulting a highly labor-intensive workflow. To overcome this challenge and facilitate the development of a high throughout assay, we developed the MPN assay in a 96-well microtiter plate format with a 10-fold reduction in test volume (i.e., 0.1 ml/well). This format was used for MPN based quantification of *Pseudomonas* spp. which reaches a high concentration of 2.55×10^{8} MPN/well within a relatively short time. The MPN quantification of *L. monocytogenes* was conducted in microcentrifuge tubes due to the necessary addition of 40 µl/ml of selective supplement after 4 hours of incubation. That would equate to 4 µl/well of the supplement added to a smaller test volume in a microtiter plate, which may cause increased pipetting error.

Future direction

Repeated testing using the current protocol to identify a possible technical error

The high level of TACC in facility F1, F2, and F3 on day 0 were not explainable since we used a non-coated microtiter plate with two PBS rinses prior to quantification of attached cells in the beginning of the experiment. We are planning to repeat the experiment using a currently established protocol to identify possible technical error that might have resulted in overestimated concentration of TACC on day 0. In this test trial, total aerobic mesophilic cell counts, TACC, *Pseudomonas* spp., *L. monocytogenes*, as well as the supernatant cells (i.e., unattached cells prior to detachment) will be quantified.

Biofilm characterization

The true biofilm formation characterization needs to include confirmation of EPS, which differentiates a biofilm from a simple cell adherence. In our study, the non-attached bacteria were removed by washing test wells twice with a sterile PBS buffer. However, the remaining attached cells were not confirmed to be imbedded in a biofilm comprised of both cells and EPS. In order to verify the true biofilm formation in a project that aims to quantify the growth of *L. monocytogenes* and *Pseudomonas* spp. in natural microbiome biofilm, it is recommended that steps to confirm EPS production be included.

Many studies have investigated the EPS production and have used different approaches to EPS identification. Study conducted by Carpentier and Chassaing (Carpentier & Chassaing, 2004) quantified the production of EPS through an enzyme-linked lectinsorbent assay (ELLA). The two lectins used in this study were concanavalin

A (ConA) and wheat germ agglutinin (WGA). The lectins were labeled with a peroxidase to allow for spectrophotometric quantification of bound EPS components. ConA binds to D-glucose and D-mannose residues, whereas WGS binds to N-acetyl-D-glucosamine and sialic acid residues. Another study conducted by Strathmann et al. (Strathmann, Wingender, & Flemming, 2002) also investigated in using ConA and WGA, however, they labelled them with fluorescein isothiocyanate or tetramethyl rhodamine isothiocyanate in combination to allow for epifluorescence microscopy-based and confocal laser scanning microscopy-based detection. They used this approach to characterize polysaccharides in a biofilm formed by *Pseudomonas aeruginosa*. A more recent study conducted by Henry et al. (Henry, Jessop, & Peeples, 2017) characterized the formation of biofilm by extracting EPS from the detached biomass. Follow-up analysis is achieved through Raman spectrometry; the Raman peak intensity can be used to differentiate each different molecules in the biofilm matrix by identifying the chemical bonds present (Henry et al., 2017).

In our study, it is more important to detect biofilm qualitatively rather than quantitatively. Therefore, it might be better to use lectin conjugates with molecular probes to detect the production of EPS in the biofilms.

Investigation of the efficacy of the biocontrol agents in inhibiting *L. monocytogenes* growth in a biofilm

Once the method for quantification of *L. monocytogenes* is optimized, it can be used for assessment of the effects of putative biocontrol strains on the growth of *L. monocytogenes* in the natural microbiome biofilm. Furthermore, the assay may be used to

test the efficacy of disinfecting chemicals against foodborne pathogens like *L. monocytogenes* in a complex environment. The application of biocontrol methods has attracted more attention in recent years as a pathogen control strategy complementary to chemical sanitizing interventions. The advantage of introduction of biocontrol strains is that they work through competition of available nutrients (Leriche & Carpentier, 2000), production of secondary metabolites (Ammor et al., 2006) or the production of enzymes that are lytic to cell wall in low access, difficult-to-clean areas in equipment and food processing environment (Saligkarias et al., 2002).

A number of studies has investigated the efficacy of using probiotic bacterial strains to inhibit the growth of *L. monocytogenes*. A study conducted by Maragkoudakis et al. demonstrated that *Enterococcus faecium* PCD71 and *Lactobacillus fermentum* significantly reduce the growth of *L. monocytogenes* and *S. enteritidis* when inoculated together on raw chicken, indicating the potential of these two strains to be applied as protective culture in the food products (Maragkoudakis et al., 2009). However, the spoilage potential of such biocontrol isolates also need to be assessed before use. Another study showed that *L. lactis* 368 (bac-), *Lactobacillus curvatus* MBSa3(bac+), *and Lactobacillus sakei* MBSa1 (bac+) can reduce pathogens including *L. monocytogenes* by more than 6 logs (Gómez, Ramiro, Quecan, & de Melo Franco, 2016). The potential for applying protective cultures in the food processing environments to control *L. monocytogenes* have also been evaluated. For example, Zhao et al. have successfully applied two previously *in vitro* characterized protective strains of *Lactococcus lactis subsp. Lactis C-1-92* and *Enterococcus durans* 152 (Zhao et al., 2004), to inhibit *L.*

monocytogenes growth in the floor drains of a poultry processing plant (Zhao et al., 2006). Because these strains were isolated from the food processing environment, they were more likely to establish themselves in the same environment and thus better suited to use as biocontrol agents. In our future work, we could inoculate these two strains in the natural microbiomes together with *L. monocytogenes* to measure their effect on the ability of *L. monocytogenes* to grow in a microbiome biofilm. Furthermore, we could compare the reduction efficacy achieved by biocontrol strains with that achieved by chemical sanitizers.

Further studies in this direction will provide more data that will allow for the assessment of the feasibility of biocontrol strain implementation as pathogen control interventions. However, it is necessary to carefully assess potential risks of implementation of biocontrol strains in food processing environments which could include negative effects on food quality. Furthermore, such measures could mistakenly lead some food processors to replace good cleaning and sanitizing practices with the use of biocontrol strains. Hence, it will be important to accompany implementation of such methods with appropriate education of end users.

Appendix B

Downloadable workflow file is available on the following link: https://github.com/kovaclab/Apple-packing-house-environmental-microbiomes/blob/master/Tan%20et%20al.%202019%20data%20anaylsis%20workflow.R

#Data analysis workflow

#Xiaoqing Tan

#xvt5028@psu.edu

#The Pennsylvania State University

#Microbiome sequence data analyses (carried out in Mothur v 1.39.5)

#From the raw sequencing files (.fastq)

#Set the inputdirectory and number of processors

#Making.files which include list of samples and the sequences associated with those samples (paired R1, R2)

mothur > make.file(inputdir=., type=fastq, prefix=16s)

#Combine the paired-end reads together

#Extract the sequence and quality score data from fastq files, create the contigs

mothur > make.contigs(file=16s.files)

#Summary.seqs provide descriptive statistics of all sequences in .fasta file ##

mothur > summary.seqs(fasta=16s.trim.contigs.fasta)

#Filter sequences

##Remove any sequences with ambiguous bases ("N"), shorter than 292, longer than 292

mothur > screen.seqs(fasta=16s.trim.contgis.fasta,

group=16s.contigs.groups, summary=16s.trim.contigs.summary,

minlength=292, maxlenth=292, maxambig=0)

#Remove identical (grouped) sequences; representative sequence will be picked and stored in .fasta and corresponding sequences will be saved as sequence names to reducw computational work

mothur > unique.seqs(fasta=16s.trim.contigs.good.fasta)

#Create a count table of current unique sequences

mothur > count.seqs(name=16s.trim.contigs.good.names,

group=16s.contigs.good.groups)

#Analyze the target 16S rRNA region (V4)

#Customize database to target V4 region

#Define start and end positions within a 16S rRNA sequence

#Set keepdots=F to false to remove output trailing dots from fragments

mothur > pcr.seqs(fasta=silva.nr_v132.align, start=11894, end=25319,

keepdots=F, processors=8)

#Rename reference files

mothur > rename.file(input = silva.bacteria.pcr.fasta, new=

silva.v4.fasta)

#Align the target region to silva reference database

#Ksize can be determined (ksize=); default is 8

#Allow reverse matching to the reference using flip=T

```
mothur > align.seqs(fasta=16s.trim.contigs.good.unique.fasta,
                reference=silva.v4.fasta, flip=T)
#Provide descriptive statistics of all sequences in .fasta file using summary.seqs
mothur > summary.seqs(fasta=16s.trim.contigs.good.unique.align,
                 count=16s.trim.contigs.good.count_table)

#Remove sequences that are before or after the sites of alignment from the previous step
mothur > screen.seqs(fasta=16s.trim.contgis.good.unique.align,
                group=16s.contigs.good.count_table,
                summary=16s.trim.contigs.good.unique.summary, minlength=Undecided,
                maxlenth=Undecided, maxhomop=8)

#Remove overhangs
#Remove the alignment characters that only consist of "-", using vertical=T
#Remove the sequences containing '.' by using trump=.
mothur > filter.seqs(fasta=16s.trim.contigs.good.unique.good.align,
            vertical=T, trump=.)

#Rerun unique.seqs in case new redundant sequences were created by filtering
mothur > unique.seqs(fasta=16s.trim.contigs.good.unique.good.filter.fasta,
                count = 16s.trim.contigs.good.good.count_table)

#De-noise sequences
#Diffs=2 means threshold of mismatches in the sequence
mothur > pre.cluster(fasta=16s.trim.contigs.good.unique.good.filter.unique.fast
                a, count=16s.trim.contigs.good.unique.good.filter.count_table,
                diffs=2)
```

```
#Read a fasta and count file to chimera sequences
mothur > chimera.vsearch(fasta=16s.trim.contigs.good.unique.good.filter.unique.p
                recluster.fasta,
                count=16s.trim.contigs.good.unique.good.filter.unique.precluster.count
                _table, dereplicate=t)

#Remove chimera
mothur > remove.seqs(fasta=16s.trim.contigs.good.unique.good.filter.unique.prec
              luster.fasta,
              accnos=16s.trim.contigs.good.unique.good.filter.unique.precluster.deno
              vo.vsearch.accnos)

#Assign taxonomy
mothur > classify.seqs(fasta=16s.trim.contigs.good.unique.good.filter.unique.pr
                ecluster.pick.fasta,
                count=16s.trim.contigs.good.unique.good.filter.unique.precluster.denov
                o.uchime.pick.count_table, reference=silva.nr_v123.align,
                taxonomy=silva.nr_v123.tax)

#Remove chloroplast and mitochondria
mothur > remove.lineage(fasta=16s.trim.contigs.good.unique.good.filter.unique.p
                recluster.pick.fasta,
                count=16s.trim.contigs.good.unique.good.filter.unique.precluster.denov
                o.uchime.pick.count_table,
                taxonomy=16s.trim.contigs.good.unique.good.filter.unique.precluster.pi
                ck.nr_v132.wang.taxonomy, taxon=Chloroplast-Mitochondria-unknown-
```

Eukaryota)

#Calculate uncorrected pairwise distances between aligned DNA sequences. By default, a gap is only penalized; cutoff value indicates that distances larger than 0.03 (>97% similarity) will not be saved

mothur > dist.seqs(fasta=16s.trim.contigs.good.unique.good.filter.unique.preclu

ster.pick.pick.fasta, cutoff=0.03)

#Assign sequences to OTUs, Mothur provides three different methods of alignment. By default,opticlust method is used.

mothur > cluster(column=16s.trim.contigs.good.unique.good.filter.unique.preclus

ter.pick.pick.dist, count =

16s.trim.contigs.good.unique.good.filter.unique.precluster.denovo.vsea

rch.pick.pick.count_table)

#Determine how many sequences are in each OTU at the 0.03 cutoff level. Distribute OTUs into groups

#The output shared file is used as an OTU table

mothur > make.shared(list=16s.trim.contigs.good.unique.good.filter.unique.precl

uster.pick.pick.opti_mcc.list,

count=16s.trim.contigs.good.unique.good.filter.unique.precluster.denov

o.vsearch.pick.pick.count_table, label=0.03)

#Determine taxonomy for all OTUs. Outcome of this command is a taxonomy file

mothur > classify.otu(list=16s.trim.contigs.good.unique.good.filter.unique.prec

luster.pick.pick.opti_mcc.list,

count=16s.trim.contigs.good.unique.good.filter.unique.precluster.denov

o.vsearch.pick.pick.count_table,

taxonomy=16s.trim.contigs.good.unique.good.filter.unique.precluster.pick.pds.wang.pick.taxonomy, label=0.03)

####Downstream analaysis###

#Barplot showing L.*monocytogenes* occurrence in three facilities

#Load the file containing data about facility and L.*monocytogenes* occurrence

lm <- read.csv(file.choose(), header = T)

lmoccurrence <- ggplot(data = lm, aes(x=Facility, y=Number.of.samples,fill=L.*monocytogenes*))+
  geom_bar(stat = "identity",width = 0.5)+
  geom_text(aes(label=Number.of.samples, size=3), hjust=0.5, vjust=3) +
  theme_bw(base_size = 12)+
  theme(legend.text=element_text(size=15), legend.title= element_text(size=15)) +
  theme(axis.text.x = element_text(size=13), axis.text.y = element_text(size=13)) +
  theme(axis.title = element_text(size=15)) +
  scale_x_discrete(name = "Facility") +
  scale_y_discrete(name = "Number of Samples")

ggsave("lmoccurrence.pdf", plot = lmoccurrence, device="pdf", width=10, height=7, units="in",dpi=600)

#Beta diversity (PCoA) plot

#Obtain required R packages

library(phyloseq)

library(ape)

library(vegan)

```
library(ggplot2)

#Import data
set.seed(336)
otus <- import_mothur(mothur_shared_file= file.choose())
otus2 <- as.data.frame(otus)
otus.t <- t(otus)
min(rowSums(otus.t))
otus.r <- rrarefy(otus.t,4501)
OTU <- otu_table(otus.r , taxa_are_rows=FALSE)

taxon <- import_mothur(mothur_constaxonomy_file = file.choose())
taxon <- as.data.frame(taxon)
colnames(taxon) <- c("Domain", "Phylum", "Class", "Order", "Family", "Genus")
TAX = tax_table(as.matrix(taxon))
metadat <- read.table(file.choose(), sep=",", header=T, row.names=1)

META = sample_data(metadat)

#PCoA for rarefied samples (16S rRNA data)
phyloseq = phyloseq(OTU, TAX, META)
TREE = rtree(ntaxa(phyloseq), rooted=TRUE, tip.label = taxa_names(phyloseq))
phyloseq = phyloseq(OTU,TAX,META,TREE)
phyloseq

ord = ordinate (phyloseq, "PCoA", "unifrac", weighted = TRUE)
po = plot_ordination(phyloseq, ord, color="Facility",shape="L.monocytogenes")
```

```
PCOA16S <- po +
  geom_point(size=4)+theme_classic() +
  theme(legend.text=element_text(size=13), legend.title= element_text(size=15)) +
  theme(axis.text.x = element_text(size=13), axis.text.y = element_text(size=13)) +
  theme(axis.title = element_text(size=15)) +
  scale_x_continuous(name = "PC1 (10.7%)") +
  scale_y_continuous(name = "PC2 (6.6%)")

PCOA16S

#Import data for ITS
set.seed(336)
otus_ITS <- import_mothur(mothur_shared_file= file.choose())
otus2_ITS <- as.data.frame(otus_ITS)
otus.t_ITS <- t(otus_ITS)
min(rowSums(otus.t_ITS))
otus.r_ITS <- rrarefy(otus.t_ITS,5323)
OTU_ITS <- otu_table(otus.r_ITS , taxa_are_rows=FALSE)

taxon_ITS <- import_mothur(mothur_constaxonomy_file = file.choose())
taxon_ITS <- as.data.frame(taxon_ITS)
colnames(taxon_ITS) <- c("Domain", "Phylum", "Class", "Order", "Family", "Genus")
TAX_ITS = tax_table(as.matrix(taxon_ITS))
metadat_ITS <- read.table(file.choose(), sep=",", header=T, row.names=1)

META_ITS = sample_data(metadat_ITS)
```

```
#PCoA for rarefied samples (ITS data)

phyloseq_ITS = phyloseq(OTU, TAX, META)

TREE_ITS = rtree(ntaxa(phyloseq_ITS), rooted=TRUE, tip.label =
taxa_names(phyloseq_ITS))

phyloseq_ITS = phyloseq(OTU,TAX,META,TREE_ITS)

phyloseq_ITS


ord_ITS = ordinate (phyloseq_ITS, "PCoA", "unifrac", weighted = TRUE)

po_ITS = plot_ordination(phyloseq_ITS, ord_ITS,
color="Facility",shape="L.monocytogenes")

PCOAITS <- po_ITS +

  geom_point(size=4)+theme_classic() +

  theme(legend.text=element_text(size=13), legend.title= element_text(size=15)) +

  theme(axis.text.x = element_text(size=13), axis.text.y = element_text(size=13)) +

  theme(axis.title = element_text(size=15)) +

  scale_x_continuous(name = "PC1 (43.1%)") +

  scale_y_continuous(name = "PC2 (20.5%)")


PCOAITS


#Combine the 16S rRNA and ITS PCoA plots

a = plot_grid(PCOA16S + theme(legend.position= "none") , PCOAITS +
theme(legend.position = "none") ,

          ncol=1, nrow=2, labels=c("A", "B"), label_size = 20)

b = get_legend(PCOAITS)

c = plot_grid(a, b, ncol=2, rel_widths = c(3,1))

c
```

```
ggsave("PCOAcombined.pdf", plot =c, device="pdf", width=10, height=10, units="in",dpi=600)

ggsave("PCOAcombined.png", plot =c, device="png", width=10, height=10, units="in",dpi=600)


#Stack barplot for bacterial and fungal communities at a family level

#Use phyoseq objects for 16S rRNA and ITS


#Melt to long format (for ggploting)

family_16 <- phyloseq %>%

  tax_glom(taxrank = "Family") %>%                    #agglomerate at a family level

  transform_sample_counts(function(x) {x/sum(x)} ) %>%  #Transform to relative abundance

  psmelt() %>%                                  #Melt to long format

  arrange(Family)                               #Sort data frame alphabetically by phylum


family_ITS <- phyloseq_ITS %>%

  tax_glom(taxrank = "Rank5") %>%

  transform_sample_counts(function(x) {x/sum(x)} ) %>%

  psmelt() %>%

  arrange(Family)


write.csv(family_16, "combined_family_16.csv")

write.csv(family_ITS, "combined_family_ITS.csv")        #Write the filtered file into csv format


#Filter the 'Abundance' column to 'less than' 0.10 (in Excel)

#All of the outcome rows are representative families with abundance lower than 0.10
```

```
#Change all column labels under 'Family' to "Other"

#Save as .csv file

#Open file in R


#Figure 1: Facility vs. L. monocytogenes

combined_family_16s <- read.csv(file.choose(), sep=",", header=T, row.names=1)

Family_colors <-
c("#FFF5F0","#525252","#CB181D","#99000D","#EF3B2C","#FB6A4A","#FC9272","
#FEE0D2","#4292C6","#084594","#FFF5F0","#EF3B2C","#9ECAE1","#C6DBEF","#D
EEBF7","#6BAED6","#2171B5","#737373")


#Plot Figure 1

fig1 <- ggplot(combined_family_16s, aes(x = SampleOrder, y = Abundance , fill =
Family))  +

  facet_grid(Facility~Lmono)+

  geom_bar(stat = "identity") +

  scale_fill_manual(values = Family_colors) +

  theme(legend.text=element_text(size=13), legend.title= element_text(size=15)) +

  theme(axis.title.x = element_blank(), axis.text.x=element_blank(),
axis.ticks.x=element_blank(),

        axis.title.y=element_text(size=15)) +

  guides(fill = guide_legend(reverse = FALSE, keywidth = 1, keyheight = 1, ncol=1)) +

  ylab("Relative Abundance") + theme(panel.background =
element_rect(fill="transparent", color =NA),

                                     plot.background = element_rect(fill="transparent", color =NA)) +

  theme(strip.background= element_blank(), strip.text = element_text(size=15),

        panel.border = element_rect(color="black", fill=NA))


fig1
```

```
ggsave("figure1.pdf", plot =fig1, device="pdf", width=8, height=5, units="in",dpi=600)



#Figure 2: Facility vs. section

combined_family_16s <- read.csv(file.choose(), sep=",", header=T, row.names=1)

Family_colors <-
c("#FFF5F0","#525252","#CB181D","#99000D","#EF3B2C","#FB6A4A","#FC9272","
#FEE0D2","#4292C6","#084594","#FFF5F0","#EF3B2C","#9ECAE1","#C6DBEF","#D
EEBF7","#6BAED6","#2171B5","#737373")


#Plot Figure 2

fig2 <- ggplot(combined_family_16s, aes(x = SampleOrder, y = Abundance , fill =
Family))  +

  facet_grid(Facility~Section)+

  geom_bar(stat = "identity") +

  scale_fill_manual(values = Family_colors) +

  theme(legend.text=element_text(size=13), legend.title= element_text(size=15)) +

  theme(axis.title.x = element_blank(), axis.text.x=element_blank(),
axis.ticks.x=element_blank(),

        axis.title.y=element_text(size=15)) +

  guides(fill = guide_legend(reverse = FALSE, keywidth = 1, keyheight = 1, ncol=1)) +

  ylab("Relative Abundance") + theme(panel.background =
element_rect(fill="transparent", color =NA),

                                     plot.background = element_rect(fill="transparent", color =NA)) +

  theme(strip.background= element_blank(), strip.text = element_text(size=15),

        panel.border = element_rect(color="black",fill=NA))


fig2

ggsave("figure2.pdf", plot =fig1, device="pdf", width=8, height=5, units="in",dpi=600)
```

```
ggsave("figure2.png", plot =fig1, device="png", width=8, height=5, units="in",dpi=600)


library(cowplot)

a = plot_grid(fig1 + theme(legend.position= "none") , fig2 + theme(legend.position =
"none") ,

          ncol=1, nrow=2, labels=c("A", "B"), label_size = 20)

b = get_legend(fig2)

c = plot_grid(a, b, ncol=3, rel_widths = c(10,1))

c


ggsave("figre_combine.pdf", plot=c, device="pdf", width=10, height=7, units="in",
dpi=600)

ggsave("figre_combine.png", plot=c, device="png", width=10, height=7, units="in",
dpi=600)



#Make stack barplot for ITS data

combined_family_ITS <- read.csv(file.choose(), sep=",", header=T, row.names=1)

Family_colors <- c("#084594", "#2171B5",
"#4292C6","#9ECAE1","#FFF5F0","#C6DBEF",
"#DEEBF7","#6BAED6" ,"#99000D","#EF3B2C","#FC9272","#F7FBFF","#FB6A4A",
"#FCBBA1", "#FEE0D2","#525252","#737373","#CB181D","#FFDAB9","#E6E6FA")


#Plot facility vs. L.monocytogenes

ITSfacilitystack <- ggplot(combined_family_ITS, aes(x = SampleOrder, y = Abundance,
fill = Family))  + facet_grid(Facility~L.monocytogenes)+

  geom_bar(stat = "identity") +

  geom_bar(stat = "identity") +

  scale_fill_manual(values = Family_colors) +

  theme(legend.text=element_text(size=13), legend.title= element_text(size=15)) +
```

```
  theme(axis.title.x = element_blank(), axis.text.x=element_blank(),
axis.ticks.x=element_blank(),
        axis.title.y=element_text(size=15)) +
  guides(fill = guide_legend(reverse = FALSE, keywidth = 1, keyheight = 1, ncol=1)) +
  ylab("Relative Abundance") + theme(panel.background =
element_rect(fill="transparent", color =NA),
                                     plot.background = element_rect(fill="transparent", color =NA)) +
  theme(strip.background= element_blank(), strip.text = element_text(size=15),
        panel.border = element_rect(color="black", fill=NA))


#Plot Facility vs. Sections
ITSsectionstack <- ggplot(combined_family_ITS, aes(x = SampleOrder, y = Abundance,
fill = Family))  + facet_grid(Facility~Section) +
  geom_bar(stat = "identity") +
  scale_fill_manual(values = Family_colors) +
  theme(legend.text=element_text(size=13), legend.title= element_text(size=15)) +
  theme(axis.title.x = element_blank(), axis.text.x=element_blank(),
axis.ticks.x=element_blank(),
        axis.title.y=element_text(size=15)) +
  guides(fill = guide_legend(reverse = FALSE, keywidth = 1, keyheight = 1, ncol=1)) +
  ylab("Relative Abundance") + theme(panel.background =
element_rect(fill="transparent", color =NA),
                                     plot.background = element_rect(fill="transparent", color =NA)) +
  theme(strip.background= element_blank(), strip.text = element_text(size=15),
        panel.border = element_rect(color="black", fill=NA))


a_ITS = plot_grid(ITSfacilitystack + theme(legend.position= "none") , ITSsectionstack +
theme(legend.position = "none") ,
                  ncol=1, nrow=2, labels=c("A", "B"), label_size = 20)
```

```
b_ITS = get_legend(ITSfacilitystack)

c_ITS = plot_grid(a_ITS, b_ITS, ncol=3, rel_widths = c(5,1))

c_ITS


#Save and export the figure

ggsave("ITSstackcombined.pdf", plot=c, device="pdf", width=12, height=10, units="in", dpi=600)


#Making phyloseq object for rarefaction curve before normalization

phyloseq_rare_16s = phyloseq(otu_table(otus.t, taxa_are_rows=FALSE), TAX, META)

phyloseq_rare_ITS = phyloseq(otu_table(otus.t_ITS, taxa_are_rows=FALSE), TAX_ITS, META_ITS)


#Rarefaction curves

rare_16s_apple_plot <- ggrare(phyloseq_rare_16s, step = 100, se= TRUE, color="Facility")

rare_16s_byfacility_plot <- rare_16s_apple_plot + facet_grid(Facility~.) +

  theme(strip.text.y=element_blank()) +xlab("Number of OTUs") + ylab("Number of unique OTUs") +

  scale_x_continuous(breaks= seq(0,180000, 10000)) + theme(axis.text.x = element_text(size=10, angle=90)) +

  annotate("segment", x=-Inf, xend=Inf, y=-Inf, yend= -Inf) +

  annotate("segment", x=-Inf, xend=Inf, y=-Inf, yend= -Inf) +

  annotate("segment", x=-Inf, xend=Inf, y=-Inf, yend= -Inf)


rare_ITS_apple_plot <- ggrare(phyloseq_rare_ITS, step = 100, se= TRUE, color="Facility")

rare_ITS_byfacility_plot <- rare_ITS_applot_plot + facet_grid(Facility ~ .) +

  theme(strip.text.y=element_blank()) +xlab("Number of OTUs") + ylab("Number of unique OTUs") +
```

```
  scale_x_continuous(breaks= seq(0,400000, 20000)) + theme(axis.text.x =
element_text(size=10, angle=90)) +
  annotate("segment", x=-Inf, xend=Inf, y=-Inf, yend= -Inf) +
  annotate("segment", x=-Inf, xend=Inf, y=-Inf, yend= -Inf) +
  annotate("segment", x=-Inf, xend=Inf, y=-Inf, yend= -Inf)

rarefig <- plot_grid(rare_16s_byfacility_plot, rare_ITS_byfacility_plot, nrow=1, ncol=2,
labels=c("A", "B"), label_size = 20)

ggsave("rarefig.pdf", plot=rarefig, device="pdf", width=11, height=6, units="in",
dpi=600)
ggsave("rarefig.png", plot=rarefig, device="png", width=11, height=6, units="in",
dpi=600)

#Alpha diversity

alpha <-estimate_richness(phyloseq, measures=c("Shannon", "InvSimpson", "Chao1"))
estimate_richness(phyloseq, split= TRUE, measures=c("Chao1", "Shannon",
"InvSimpson"))

#Import 16S rRNA data
alpha_16s <- read.csv(file.choose(), sep = ",", header = T, row.names = 1)
alpha_ITS <- read.csv(file.choose(), sep = ",", header = T, row.names = 1)

#Pairwise.t.test for alpha diversity using Shannon and Inverse Simpson indices
pairwise.t.test(alpha_16s$Shannon, alpha_16s$Facility, p.adjust.method = "bonferroni")
pairwise.t.test(alpha_16s$InvSimpson, alpha_16s$Facility, p.adjust.method =
"bonferroni")
```

```
#Import ITS data
alpha_ITS <- read.csv(file.choose(), sep = ",", header = T, row.names = 1)

#Pairwise.t.test for alpha diversity using Shannon and Inverse Simpson indices
pairwise.t.test(alpha_ITS$Shannon, alpha_ITS$Facility, p.adjust.method = "bonferroni")
pairwise.t.test(alpha_ITS$InvSimpson, alpha_ITS$Facility, p.adjust.method =
"bonferroni")

#Violin plots for alpha diversity
library(ggpubr)

#16S alpha diversity violin plots
alpha_16s <- read.csv(file.choose(), sep = ",", header = T, row.names = 1)
alpha16s1 <- ggviolin(alpha_16s, x = "Facility", y = "Shannon", add = "boxplot",
                      fill= "Facility" ) +
  theme(axis.text.x = element_text(size=13), axis.text.y = element_text(size=13)) +
  theme(axis.title = element_text(size=15))
alpha16s2 <- ggviolin(alpha_16s, x = "Facility", y = "InvSimpson", add = "boxplot",
                      fill  = "Facility" ) +
  theme(axis.text.x = element_text(size=13), axis.text.y = element_text(size=13)) +
  theme(axis.title = element_text(size=15))


#ITS alpha diversity violinplots
alpha_ITS <- read.csv(file.choose(), sep = ",", header = T, row.names = 1)
alphaITS1 <- ggviolin(alpha_ITS, x = "Facility", y = "Shannon", add = "boxplot",
                      fill= "Facility" ) +
```

```
  theme(axis.text.x = element_text(size=13), axis.text.y = element_text(size=13)) +
  theme(axis.title = element_text(size=15))
alphaITS2 <- ggviolin(alpha_ITS, x = "Facility", y = "InvSimpson", add = "boxplot",
                      fill  = "Facility" ) +
  theme(axis.text.x = element_text(size=13), axis.text.y = element_text(size=13)) +
  theme(axis.title = element_text(size=15))



#Combined 16S and ITS alpha diversity plots
a = plot_grid(alpha16s1 + theme(legend.position= "none") ,
              alpha16s2 + theme(legend.position = "none") ,
              alphaITS1 + theme(legend.position = "none"),
              alphaITS2 + theme(legend.position = "none"),
              ncol=2, nrow=2, labels=c("A", "B","C","D"), label_size = 20)


ggsave("alphadiversity.pdf", plot =a, device="pdf", width=12, height=10,
units="in",dpi=600)
ggsave("alphadiversity.png", plot =a, device="png", width=12, height=10,
units="in",dpi=600)


#Pairwise PERMANOVA
library(devtools)
install_github("pmartinezarbizu/pairwiseAdonis/pairwiseAdonis")
library(pairwiseAdonis)


#Run pairwise PERMANOVA for 16S rRNA data
permanova_data_16s <- data.frame(sample_data(phyloseq))
```

```
pairwise_perm_16s_f <- pairwise.adonis(otu_table(phyloseq),
permanova_data_16s$Facility)

pairwise_perm_16s_s <- pairwise.adonis(otu_table(phyloseq),
permanova_data_16s$Section)


#Export the file in .csv

write.csv(pairwise_perm_16s_f, "microbiome_pairwise_Facility.csv")

write.csv(pairwise_perm_16s_s, "microbiome_pairwise_Section.csv")


#Run pairwise PERMANOVA for ITS data

permanova_data_ITS <- data.frame(sample_data(phyloseq))

pairwise_perm_ITS_f <- pairwise.adonis(otu_table(phyloseq),
permanova_data_ITS$Facility)

pairwise_perm_ITS_s <- pairwise.adonis(otu_table(phyloseq),
permanova_data_ITS$Section)


#Export the file in .csv

write.csv(pairwise_perm_ITS_f, "mycobiome_pairwise_Facility.csv")

write.csv(pairwise_perm_ITS_s, "mycobiome_pairwise_Section.csv")


#PICRUSt analysis plot

#Import csv file for picrust, actural abundance

picrustfuntion <- read.csv(file.choose(), sep = ",", header = T)


#Make boxplot based on picrust information

allfunctionabun <- ggplot(picrustfuntion, aes(x=Facility, y=Abundance,
fill=Pathway.category)) +

  theme_bw() +geom_boxplot()+

  theme(legend.text=element_text(size=13), legend.title= element_text(size=15)) +
```

```
  theme(axis.title.x = element_text(size=15),
        axis.title.y=element_text(size=15))


#Make a plot based on function abundance
functionabundance <- ggplot(picrustfuntion, aes(x=Facility, y=Abundance, fill=Pathway.category)) +theme_bw() +geom_boxplot()


#Import .csv file for picurst, relative abundance, all combined
picrustrelabun <- read.csv(file.choose(), sep = ",", header = T)
refunctionabun <- ggplot(picrustrelabun, aes(x=Facility, y=relative.abundance, fill=Category)) +theme_bw() +
  geom_boxplot() +
  theme(legend.text=element_text(size=13), legend.title= element_text(size=15)) +
  theme(axis.title.x = element_text(size=15),
        axis.title.y=element_text(size=15))


#Create a plot for functional categories based on relative abundance
re_all_plot <-ggplot(picrustrelabun, aes(x=Facility, y=relative.abundance, fill=Category)) +theme_bw() + geom_boxplot()


#Import csv file for picrust, relative abundance, by category
picrustfuntioncate <- read.csv(file.choose(), sep = ",", header = T)


#Pairwise.t.test for significant difference between categories
pairwise.t.test(picrustfuntioncate$Metabolism, picrustfuntioncate$Facility, p.adjust.method = "bonferroni")
ggplot(picrustfuntioncate, aes(x=Facility, y=Cellular.Processes)) + theme_bw() + geom_col()
```

```
#Plot metabolism and environment functional categories for each facility
metabolism <- ggplot(picrustfuntioncate, aes(x=Facility, y=Metabolism)) + theme_bw()
+
  geom_boxplot() +
  theme(legend.text=element_text(size=13), legend.title= element_text(size=15)) +
  theme(axis.title.x = element_text(size=15),
        axis.title.y=element_text(size=15))

Environment <- ggplot(picrustfuntioncate, aes(x=Facility,
y=Environmental.Information.Processing)) +
  theme_bw() + geom_boxplot() +
  theme(legend.text=element_text(size=13), legend.title= element_text(size=15)) +
  theme(axis.title.x = element_text(size=15),
        axis.title.y=element_text(size=15))

a = plot_grid(allfunctionabun, refunctionabun, nrow=2, labels=c("A", "B"), label_size =
20)
b =plot_grid(metabolism, Environment,nrow = 2, labels = c("C","D"), label_size = 20)
c = plot_grid(a, b, ncol=2, rel_widths = c(6,3))
c

ggsave("picrust.pdf", plot=c, device="pdf", width=12, height=10, units="in", dpi=600)
ggsave("picrust.png", plot=c, device="png", width=12, height=10, units="in", dpi=600)
```

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