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Title: Prevalence and Characterization of *Listeria* spp. Recovered from Pacific Northwest Produce Handling and Processing Facilities.

Abstract approved:

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Foodborne illness in the United States continues to be a complex and recurring issue despite our increased understanding of the pathogenic microorganisms responsible. Foodborne illness outbreaks and product recalls linked to pathogenic bacteria have been more frequent in the produce industry (e.g. fruits and vegetables) in the last ten years. Currently one of the most concerning foodborne bacterial pathogens in the produce industry is *Listeria monocytogenes*. This foodborne pathogen has been linked to multistate produce-associated outbreaks causing hundreds of illnesses and dozens of deaths. In several of these outbreaks, *L. monocytogenes* strains isolated from clinical patients were found to be persistent in produce handling and processing (PHP) facilities. This suggests that *L. monocytogenes* may contaminate product through cross-contamination events in PHP facilities and current food safety interventions in these environments may be inadequate to prevent transfer to food.

Since the passage of the Food Safety and Modernization Act (FSMA) in 2011, States in the Pacific Northwest (PNW) that supply the U.S. with hundreds of specialty crops have been more focused on food safety. The produce industry in the PNW needs data and knowledge to effectively control *L. monocytogenes* in PHP facilities and comply with FSMA. The objective of this study was to investigate the prevalence of *Listeria* spp. in seven PHP facilities in Oregon and Washington through environmental monitoring on non-food contact surfaces only, with emphasis on the pathogenic species *L. monocytogene*s. The facility with the highest prevalence would receive additional and more intensive environmental sampling. A secondary objective was to characterize *Listeria* spp. strains recovered from PHP facilities and group potentially related strains. Characterization of strains was done through speciation, a multiplex PCR serogrouping assay, and antimicrobial resistance (AMR) profiling. A third objective was to track related strains throughout one facility and identify potential contamination sources.

Environmental samples were collected from all PHP facilities at least twice (Rounds 1 and 2) from 2018-2019 and tested for *Listeria* spp. using a modified ISO 11290-1 method. *Listeria* spp. were not recovered from two PHP facilities (5/7, 70%). The prevalence of *Listeria* spp. through the first two rounds varied significantly across all PHP facilities and overall prevalence was relatively low (24/350, 6.9%). Additionally, *L. monocytogenes* was recovered in all PHP facilities positive for *Listeria* spp. One facility contributed >50% of the positive samples for the entire study. This facility minimally processes and packs raw produce only, does not have an environmental monitoring program and it is not subject to environmental monitoring regulations included in FSMA.

Throughout the next rounds of sampling in only this facility (Rounds C and D) *L. monocytogenes* was more frequently recovered from environmental samples (26/100, 26%). A majority of *L. monocytogenes* strains were recovered from production room drains, foot traffic and forklift traffic entry points, samples taken outside the facility and in high traffic production floor areas. Characterization and tracking suggested that *Listeria* spp. are commonly brought into this facility on the bottom of employee shoes or forklifts and subsequently deposited throughout the facility.

Serogrouping of *L. monocytogenes* strains showed that isolates from all facilities may be serotypes that are regularly associated with listeriosis foodborne illness outbreaks, serotypes 1/2a and 4b. AMR profiling, though, indicated that all recovered *Listeria* spp. strains were sensitive to antibiotics commonly used in the treatment of foodborne listeriosis. Collectively, our data suggest that there is an increased risk of environmental contamination for PHP facilities that function as packinghouses and handle multiple types of raw produce, though additional studies including diverse PNW PHF facilities are needed to support this hypothesis. Antibiotic resistance in *L. monocytogenes* food chain isolates should be continuously monitored, including further genomic characterization of isolates to better understand overall strain relatedness, pathogenicity and AMR potential.

Prevalence and Characterization of *Listeria* spp. Recovered from Pacific Northwest Produce Handling and Processing Facilities

by
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1.1 Summary of situation

Reports of foodborne illness outbreaks or recalls are present in the media daily, implicating a wide variety of foods. Throughout the 2000s, several large multistate foodborne illness outbreaks were linked to various produce types;

- 2011 Jensen Farms listeriosis outbreak linked to whole cantaloupes. 147 confirmed cases and 33 deaths (McCollum et al., 2013).
- 2014 caramel apple listeriosis outbreak originating from California apple grower. 35 confirmed cases and 7 deaths (Angelo et al., 2017).
- 2016 listeriosis outbreak associated with Dole packaged salad. Cases were confirmed throughout the United States and in Canada. 19 confirmed cases and 1 death (CDC, 2016a).

The United States (U.S.) government responded to this public health problem by taking a more holistic and thorough approach to food safety resulting in the passage of the Food Safety Modernization Act (FSMA) in 2011. Implementation of FSMA led to the Food and Drug Administration (FDA) promulgating seven primary rules. The two largest of these rules are the Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption (aka “Produce Safety Rule” (PSR)) and the Current Good Manufacturing Practice, Hazard Analysis, and Risk-based Preventive Controls for Human Food (aka “Preventive Controls for Human Food” (PCHF) Rule). Most food manufacturers

in the U.S. especially those that produce Ready-To-Eat (RTE) foods, have had to rethink components of food safety in their facilities.

Produce handling and processing (PHP) facilities have a particularly complicated arrangement with the FSMA rules. At its simplest, the PSR applies to raw agricultural commodities (RACs) from primary production operations (farms), harvesting operations, and post-harvest activities, whereas the PCHF applies "food" (no longer considered RACs) and food processing facilities. As expected, there are operations that grow and process produce and would clearly fall under both rules. However, there are many other operations where the lines are less clear. FDA has drafted the Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption: Guidance for the industry to assist operations in classifying their activities as handling or processing; however, these determinations are arbitrary and continued discourse is necessary. Classification of facilities under each rule is particularly important because of the significantly different approach and expectation of the control of environmental pathogens, particularly *Listeria monocytogenes*.

Outbreaks and research have demonstrated that *L. monocytogenes* infiltrates food manufacturing environments, establishes itself as a permanent resident of facilities, and then has the potential to contaminate subsequent production days. There is a significant body of work that has investigated the prevalence of *Listeria* spp. in dairy, meat, and seafood processing facilities. However, very few studies have investigated the presence of *L. monocytogenes* in produce operations, such as PHP facilities. This research is necessary to shape recommendations for PHP facilities, to better understand their risks, acknowledge their contamination sources, and effectively manage their operation to reduce harborage of

L. monocytogenes. Providing data on the environmental prevalence and phenotypic characteristics of *L. monocytogenes* strains from PHP facilities across this region will help the industry comply with regulation as well as to reduce the public health risk for *L. monocytogenes* in the food supply.

1.2 Foodborne illness linked to produce

According to the Center for Disease Control (CDC), each year there are around 48 million illnesses, 128,000 hospitalizations, and 3,000 deaths attributed to foodborne contamination (Scallan et al., 2011). There are seven main pathogens that account for the majority of the morbidity and mortality associated with foodborne illness. Norovirus, *Salmonella, Campylobacter* spp., and *Clostridium perfringens* account for the majority of foodborne illnesses, whereas Shiga toxin-producing *E. coli* (STEC), *L. monocytogenes*, and *Toxoplasma gondii* are associated with higher rates of hospitalizations and deaths (Scallan et al., 2011). As of 2013, 50% of acquired foodborne illnesses have been found to be produce associated, and 32% of produce-associated outbreaks result from infections caused by a bacterial foodborne pathogen (Painter et al., 2013). *L. monocytogenes* and *Salmonella* spp. account for 70% of the deaths attributed to foodborne disease (Barton Behravesh et al., 2011). The 2011 multistate listeriosis outbreak linked to cantaloupe (McCollum et al., 2013) elevated *L. monocytogenes* as one of the most concerning foodborne pathogens associated with produce (Garner and Kathariou, 2016; Sheng et al., 2017). The owners of Jensen Farms, linked to the outbreak, faced a small financial penalty and were required to do community service hours. The produce industry must respond to this risk to protect public health and the longevity of their business. Understanding the biology and

ecology of *L. monocytogenes* is critical to support the produce industry to combat and control this pathogen.

1.3 *Listeria* spp. and listeriosis

The *Listeria* genus is now composed of 20 species after the recent discovery of the species *Listeria costaricensis* (Núñez-Montero et al., 2018). The *Listeria* genus comprises a group of Gram-positive, facultative anaerobic, non-spore forming bacteria (Farber and Peterkin, 1991; Orsi and Wiedmann, 2016). The genus can be separated into two groups; *Listeria sensu stricto* and *Listeria sensu lato*. The separation of groups is based on shared genomic and phenotypic characteristics between species (Liao et al., 2017; Orsi and Wiedmann, 2016). *Listeria sensu stricto* includes the most studied and important species in the genus, *L. monocytogenes*. This species is a known foodborne pathogen that can infect humans and can cause the disease listeriosis (Clark et al., 2010; Farber and Peterkin, 1991; Swaminathan and Gerner-Smidt, 2007; Vazquez-Boland et al., 2001). *L. monocytogenes* is also the most well-known species because of its ability to survive in the natural environment as a saprophyte (survives off of decaying plant matter) and in the cells of animal and human hosts potentially causing disease (Freitag et al., 2009). The ingestion of food contaminated with *L. monocytogenes* can lead to an infection in susceptible hosts leading to a combination of symptoms termed listeriosis (Farber and Peterkin, 1991; Goldfine and Shen, 2007). *Listeria ivanovii*, another confirmed rare pathogenic species in this genus, has been associated with a few rare human listeriosis cases in individuals with compromised immune systems (Guillet et al., 2010; Snapir et al., 2006). Susceptible hosts of *L. monocytogenes* include pregnant woman, neonates, the elderly, and individuals with

compromised immune systems (Farber and Peterkin, 1991). *L. monocytogenes* infections can be non-invasive or invasive. Non-invasive infections are defined as those that display typical symptoms of gastroenteritis and do not expand beyond the digestive tract (Swaminathan and Gerner-Smidt, 2007). Non-invasive listeriosis has been observed in healthy individuals that ingest foods with high levels of *L. monocytogenes* contamination (Farber and Peterkin, 1991; Miettinen et al., 1999).

Invasive listeriosis is when the infection spreads to other systems, including the circulatory system and nervous system leading to septicemia and meningitis, respectively (Farber and Peterkin, 1991; Vazquez-Boland et al., 2001). *L. monocytogenes* is unique in its ability cross the blood-placental barriers. *L. monocytogenes* cells crossing the blood-placental barrier can infect the fetus (Vazquez-Boland et al., 2001). Invasive listeriosis in neonates leads to septicemia or meningitis, often resulting in late-term abortions.

Foodborne listeriosis is a very concerning bacterial disease, with high mortality rates (20-40%) for those with underlying conditions (Goulet et al, 2012). In comparison, infection and disease from other pathogenic bacteria such as *Salmonella* spp. and *E. coli* O157:H7 have mortality rates <1 % (FDA, 2017; Scallan et al., 2011). The high mortality rate alone makes *L. monocytogenes* an important foodborne pathogen to monitor and characterize in the food chain.

1.4 Characterization of *Listeria* spp.

In food safety, the characterization of pathogens is conducted for a wide range of reasons. These include foodborne disease surveillance, food and food manufacturing environmental pathogen testing, outbreak investigations and food technology

developments (Lakicevic et al., 2017). Researchers and medical professionals evaluate genotypic and phenotypic characteristics of pathogens in order to protect public health. *L. monocytogenes* characterization techniques are diverse. With the development, speed, and affordability of DNA sequencing technology these characterization efforts have improved drastically.

1.4.1 Serotyping and lineages

Serotyping is a subtyping method that identifies variations in antigens or antigenic components that are differentially recognized by the immune system (Henriksen, 1978). Serotyping of *L. monocytogenes*, particularly strains isolated from human listeriosis cases, food, and food manufacturing facilities provides an initial snapshot of strain diversity and human pathogenicity. *L. monocytogenes* can be identified and separated into four major lineages. Two lineages are particularly important in foodborne illness: lineage I and lineage II (Table 1.1) (Orsi et al., 2011; Piffaretti et al., 1989; Rasmussen et al., 1995; Ward et al., 2008).

Table 1.1. Summary of *Listeria monocytogenes* lineages. Adapted from Orsi et al., 2011.

	***Listeria monocytogenes* Lineages**			
	I[1]	**II**[1]	**III**	**IV**
Serotype[1]	1/2b[2], 3b, 3c, 4b[2]	1/2a[2], 1/2c[2], 3a	4a, 4b, 4c	4a, 4b, 4c
References	Piffaretti et al (1989)	Piffaretti et al (1989)	Rasmussen et al. (1995)	Ward et al. (2008)

[1]All known serotypes not present.
[2]Important lineages and serotypes in foodborne listeriosis illness.

L. monocytogenes is serotyped by the variations in the somatic (O) and flagellar (H) antigens and has been classified into at least 13 serotypes (Borucki and Call, 2003;

Doumith et al., 2004; Liu, 2006; Seeliger and Höhne, 1979). Previous studies have shown the major foodborne *L. monocytogenes* serotypes to be 1/2b and 4b in lineage I, and 1/2a and 1/2c in lineage II (Seeliger and Höhne, 1979; Tappero et al., 1995). Of those serotypes, 1/2a and 4b have been dominant in foodborne illness cases of listeriosis (Buchrieser et al., 1993; Doumith et al., 2004; Farber and Peterkin, 1991; Schönberg et al., 1996). Serotype 4b and 1/2a cause an overwhelming majority of human listeriosis illnesses and have both been isolated from food chain systems (Farber and Peterkin, 1991; Ferreira et al., 2013; Orsi et al., 2011; Sauders et al., 2009). Studies have also reported that they differ in relative importance and prevalence in outbreak scenarios and food chain systems (Clark et al., 2010; Farber and Peterkin, 1991; Ferreira et al., 2013; Orsi et al., 2011).

Historically, agglutination methods were used to type *L. monocytogenes* strains; however, these methods are expensive, time consuming, subjective, and require extensive training (Borucki and Call, 2003; Doumith et al., 2004; Jordan et al., 2014). A more recent method developed by Doumith et al. (2004) separates the four major serotypes (1/2a, 1/2b, 1/2c, and 4b) into four distinct serogroups by a multiplex PCR assay (Doumith et al., 2004). Marker genes are used to identify and separate strains into the four serogroups. This is a highly specific, simple, and cost effective method that provides valuable subtyping data, as well as a *Listeria* genus confirmation step (Doumith et al., 2004). The consistent presence of some serotypes in food or a food manufacturing environment, such as serotypes 1/2a and 4b, could indicate a public health concern.

1.4.2 Molecular characterization techniques for Listeria *spp.*

Molecular tools are valuable for subtyping and differentiating *Listeria* spp. strains. The use of these tools is common in the identification and tracking of foodborne outbreaks. For many years, pulsed field gel electrophoresis (PFGE) served as the gold standard for strain identification in outbreak investigations. PFGE is the separation of restriction enzyme digested genomic DNA that results in a "fingerprint" pattern. These patterns can be compared for different isolates and provides a high level of discrimination (Fox et al., 2012; Revazishvili et al., 2004).

Multi-locus sequence typing (MLST) compares differences in genetic fragments of housekeeping genes across *L. monocytogenes* isolates (Revazishvili et al., 2004b; Salcedo et al., 2003). Changes in housekeeping genes is typically a slow and stable process, resulting in a high potential for relatedness between strains with similar MLST profiles (Enright and Spratt, 1999; Salcedo et al., 2003).

Whole genome sequencing (WGS) is the analysis of the genomic DNA sequence of any organism. This approach allows for the identification and comparison of isolates at the single nucleotide level or the identification of gene or operon insertions or deletions. Genome assemblies and annotations are often based on reference strains within a database. Databases for foodborne pathogens are increasing in number, and their functionality continues to improve. An example is the GenomeTrakr Network supported by Food and Drug Administration (FDA) and the National Center for Biotechnology Information (NCBI) Pathogen Detection system. GenomeTrakr has thousands of pathogen genomic sequences from food, environmental sources, and patients. As WGS has a relatively quick turnaround time, it is becoming more cost efficient and readily available

each year. This tool has been replacing most of the previously relied-on characterization methods. As a one-step characterization method for pathogens, among other things, WGS can be used to identify, subtype, detect pathogenic markers, and make predictions on antimicrobial resistance profiles.

L. monocytogenes is by far the most sequenced species in the *Listeria* genus. GenomeTrakr has sequenced more 28,000 *L. monocytogenes* isolates to date. The genome of *L. monocytogenes* is approximately 2.9 Mb(Glaser et al., 2001) and includes a large number of genetic components that explain its unique ability to adapt and survive in diverse environments (i.e. water, soil, food manufacturing environments). WGS analysis between *L. monocytogenes* outbreak strains have revealed high levels of relatedness (Burall et al., 2017).

Subtyping networks, such as PulseNet and the Institut Pasteur BIGSdb-*Lm* allows for global and nationwide comparability of pathogenic strains based on profiles created by subtyping methods (Swaminathan et al., 2001). As of 2019, PulseNet no longer uses PFGE data and has completely switched to the use of WGS for *Listeria* spp.

1.4.3 Antimicrobial resistance

Two million people in the U.S. become infected with antimicrobial resistant (AMR) pathogenic bacteria and around 23,000 people die each year from AMR infections (CDC 6). The use of antibiotics relevant to human health in animal production has been shown as a contributor to AMR resistance in foodborne pathogens (White, 2002). AMR zoonotic pathogens like *L. monocytogenes* have been shown to be transmitted to humans through the contamination of food (White, 2002). This type of transfer may be more likely to occur

at PHP environments that are also located near livestock operations. Studies have shown that produce-associated illnesses have originated from animal hosts, shedding the pathogen into the environment, and the environment serving as a reservoir for the contamination of foods (Bruggen et al., 2008; Jang, et al., 2014). The potential severity of listeriosis (Farber and Peterkin, 1991; Vazquez-Boland et al., 2001) and increased produce-associated *L. monocytogenes* outbreaks and recalls (Garner and Kathariou, 2016; Zhu et al., 2017) warrant the monitoring of AMR for any *Listeria spp.* food chain isolate.

1.5 Food safety outbreaks and recalls

1.5.1 Overview

It seems that recalls and outbreaks linked to foodborne pathogens in foods have become more frequent in the U.S. in recent years. There are several factors that may contribute to this increase that are not due to unsafe food systems or any actual statistically significant increase. The risk of contracting a foodborne illness from a pathogenic bacteria is greater for high risk populations, such as the elderly (Barton Behravesh et al., 2011; Smith, 1998). Since the 1970s, life expectancy has increased for all genders and races in the U.S. (CDC, 2017a), resulting in a large portion of the population that falls into the category of being at risk for contracting a foodborne illness. Increased testing for pathogenic organisms may also be contributing to this noticeable increase in food recalls. Food manufacturing facilities may now have increased pathogen testing of their product or swab samples from their manufacturing environment. This may be required by customers or by new government regulations under FSMA. For example, under the "Code of Federal Regulation, Current Good Manufacturing Practice, Hazard Analysis, and Risk-based

Preventive Controls for Human Food, Section 117.165 Verification of Implementation and Effectiveness of Environmental Monitoring, Sampling and Testing the Production Environment", environmental monitoring for an environmental pathogen [such as *L. monocytogenes*] or for an appropriate indicator organism [e.g., *Listeria* spp.] is required if contamination of a RTE food is a hazard requiring a preventive control.

1.5.2 Listeriosis outbreaks and Listeria *spp. recalls in produce*

Table 1.2 provides a list of known produce-associated listeriosis outbreaks in the U.S. The first two recorded listeriosis outbreaks were produce-associated, one in the U.S. in 1979 and one in Canada in 1981. The first suspected outbreak of listeriosis was in Boston and linked to raw vegetables (celery, lettuce and tomatoes) (Garner and Kathariou, 2016; Ho et al., 1986). The earliest confirmed listeriosis foodborne illness outbreak was in Canada in 1981, and it was produce-associated (Schlech et al., 1983). The outbreak was linked to cabbage contaminated with a *L. monocytogenes* serotype 4b strain, leading to 41 listeriosis cases and 18 deaths (Garner and Kathariou, 2016). In subsequent years, listeriosis outbreaks were more often linked foods of animal origin (meat, hot dogs, soft cheeses, etc) (Garner and Kathariou, 2016).

Table 1.2. Outbreaks of listeriosis in produce in the United States[1, 2].

Year	Location	No. cases (deaths)	Produce type	Reference
1979	Boston	20 (3)	Multi-vegetable	Ho et al.
2008	Multistate	20 (0)	Sprouts	CDC 2008
2010	Texas	10 (5)	Celery	Gaul et al.
2011	Multistate	147 (33)	Whole cantaloupes	McCollum et al.
2014	Multistate	2 (1)	Stone fruits	Jackson et al.
2014	Multistate	5 (2)	Mung bean sprouts	CDC 2014
2014	Multistate	35 (7)	Caramel apples	Angelo et al.
2016	Multistate	19 (1)	Packaged salads	CDC 2016
2016	Multistate	9 (3)	Frozen vegetables	CDC 2016

[1]Adapted from *Listeria monocytogenes* in Fresh Produce: Outbreaks, Prevalence and Contamination Levels, Zhu et al., 1986.

[2]Adapted from Fresh Produce-Associated Listeriosis Outbreaks, Sources of Concern, Teachable Moments, and Insights, Garner and Kathariou, 2016.

In 2011, the U.S. saw one of its worst foodborne outbreaks when whole cantaloupe from Jensen Farms was contaminated with *L. monocytogenes* (McCollum et al., 2013). The outbreak affected 28 states and was unique in that it involved a whole intact fruit as a vehicle, as opposed to chopped or cut produce. It also had two outbreak strains, with two different serotypes, 1/2a and 1/2b (Garner and Kathariou, 2016; McCollum et al., 2013). This outbreak caused more deaths (n=33) from any foodborne illness outbreak in the U.S. in over 80 years (Desai et al., 2019). The Jensen Farms outbreak changed the narrative for listeriosis outbreaks and brought increased attention to the connection between *Listeria* spp. and produce. Another unique produce-associated listeriosis outbreak occurred in 2014 with apples as the vehicle (Angelo et al., 2017). Illnesses were associated with the consumption of pre-sliced, whole apples, and caramel apples (Angelo et al., 2017; Garner

and Kathariou, 2016). In total, 35 case were confirmed across 12 states and seven people died (Angelo et al., 2017).

The diversity and complexity of listeriosis outbreaks in the U.S. have established that diverse types of produce may be vehicles for *L. monocytogenes* infection (Angelo et al., 2017). This has led to increased regulatory testing of various food products for *L. monocytogenes* that may not have been previously identified as vehicles for the organism. Regulatory pressure has also motivated the food industry to implement testing requirements into their supply chain. This increased testing has led to an increase in detection and an increase in recalls. The recall may be the result of a positive finding of *Listeria* spp. or *L. monocytogenes* in the product, or due to a positive result in a sample taken from the production environment, specifically food contact surfaces. A list of produce and produce-associated *Listeria* recalls from 2016-2019 is provided in Table 1.3.

Two of the largest recalls in recent years occurred in 2016 and 2017. In April of 2016, CRF Frozen foods recalled 11 frozen vegetable due to a possible contamination with "*Listeria*", with *L. monocytogenes* not specifically mentioned in notifications initially (CDC, 2016b). CRF continued to expand its recall into other products including all frozen organic and traditional fruit and vegetable products. The Centers for Disease Control and Prevention (CDC) reported that nine people across multiple states from September 2013 to May 2016 fell ill with listeriosis due to this recalled product. Products recalled from this event can be tracked in Table 1.3 as CRF2016 in the recall reason column.

In October 2017, Mann Packing issued a voluntary recall linked to minimally processed vegetable products (CDC, 2017b). The recall was due to a single positive on a product found by the Canadian Food Inspection Agency (CDC, 2017b). No one became ill

from this recall, yet, nearly every day in the last weeks of October 2017 a company that contained Mann vegetable products released a recall statement. The companies and products associated with the Mann's recall can be tracked in Table 1.3 as Mann2017 in the recall reason column.

Table 1.3. United States Recalls, Market Withdrawals, & Safety Alerts | FDA. *Listeria* spp. and produce related products, 2016 to 2019[1, 2].

Date	Brand Name(s)	Product Description	Recall Reason	Company
7/15/2019	Green Giant, Growers Express, others	Fresh vegetable products	*Listeria monocytogenes*	Growers Express
7/1/2019	Green Giant, Growers Express, others	Butternut squash, cauliflower, zucchini, and veggie bowl products	*Listeria monocytogenes*	Growers Express
6/19/2019	Woodstock	Organic grilled red peppers	*Listeria monocytogenes*	UNFI
6/18/2019	Signature Select	Avocado chunks	*Listeria monocytogenes*	Nature's Touch Frozen Foods West, Inc.
6/17/2019	Sprouts Farmers Market	Frozen cut leaf spinach	*Listeria monocytogenes*	Sprouts Farmers Market
3/25/2019	Henry Avocado Corporation	California organic avocados	*Listeria monocytogenes*	Henry Avocado Corporation
3/8/2019	Fullei Fresh	Organic bean sprout	*Listeria monocytogenes*	Fullei Fresh
3/4/2019	Marketside	Green beans and butternut squash	*Listeria monocytogenes*	Southern Specialties Inc.
2/1/2019	Jac. Vandenberg, Inc.	Peaches, nectarines, plums	*Listeria*	Jac. Vandenberg, Inc.
1/31/2019	Dole, Fresh Selections, Simple Truth, others	Packaged salads	Continuation of 2016 *Listeria* outbreak	Dole
12/15/2018	Eat Smart, Salad Shake Ups	Salads	*Listeria monocytogenes*	Apio, Inc.
5/25/2018	Private Brand	Frozen broccoli cuts	*Listeria*	Giant Food LL, Stop & Shop
3/17/2018	Oregon Food Bank	Pumpkin Seeds	*Listeria monocytogenes*	Oregon Food Bank
2/9/2018	Edamame (no brand name)	Edamame	*Listeria*	Advanced Fresh Concepts Franchise Corp.
2/9/2018	Season's Choice	Sweet peas	*Listeria*	Lakeside Foods, Inc.
2/8/2018	Sunmba	Frozen ajiaco (vegetables mix)	*Listeria monocytogenes*	Barberi International Inc.
2/8/2018	Southeastern grocers, Publix Inc.,	Fajita blend, stir fry vegetable, vegetable	*Listeria monocytogenes*	Country Fresh Orlando, LLC

Date	Brand Name(s)	Product Description	Recall Reason	Company
2/8/2018	Great Value	Frozen organic dark sweet pitted pitted cherry products	*Listeria monocytogenes*	SunOpta Inc./Sunrise Growers Inc.
2/8/2018	CC Kitchens	Salad and slaw kits containing leafy greens	*Listeria*	CC Kitchens
2/8/2018	Peak, others	Spinach	*Listeria*	Horton, others
2/7/2018	Season's Choice	Frozen peas	*Listeria monocytogenes*	Lakeside Foods
2/6/2018	Choice Farms	Stuffed mushrooms	*Listeria monocytogenes*	Choice farms LLC
2/6/2018	Veggie Noodle Co	Butternut Spirals	*Listeria monocytogenes*	Veggie Noodle Co
12/29/2017	Nature's Touch	Frozen green beans	*Listeria monocytogenes*	Nature's Touch Frozen Foods, LLC
12/29/2017	Meijer	Package products containing apple slices	*Listeria monocytogenes*	Meijer
12/29/2017	Apple Ridge	Gala, fuji, honeycrisp and golden delicious apples	*Listeria monocytogenes*	Jack Brown Produce, Inc.
12/22/2017	Fresh Pak, Michigan, Aunt Mid's	Packaged products containing apple slices	*Listeria monocytogenes*	Fresh Pak, Inc.
11/7/2017	Nature's Touch	Frozen Green Beans	*Listeria monocytogenes*	Nature's Touch Frozen Foods LLC
10/25/2017	CP Fresh	Salad kits and stir fry mixes	*Listeria monocytogenes,* Mann2017	Triple B Corporation
10/25/2017	Charlie's Produce, Alaska Carrot	Salad kits and stir fry mixes	*Listeria monocytogenes,* Mann2017	Triple B Corporation
10/24/2017	Albertsons, Safeway Vons, Pak'N Save	Fresh vegetable trays and cups	*Listeria monocytogenes*	Albertsons, Safeway Vons, and Pak'N Save
10/24/2017	Albertsons, Safeway Vons, Pak'N Save	Fresh vegetable trays and cups	*Listeria monocytogenes*	Albertsons, Safeway Vons, and Pak'N Save
10/23/2017	Pacific Coast Fruit Company	Bagged Processed Salads	*Listeria monocytogenes,* Mann2017	Pacific Coast Fruit Company
10/23/2017	Just Cut	Broccoli Florets	*Listeria monocytogenes,* Mann2017	Paragon Wholesale Foods Corp.
10/23/2017	King Soopers, City Market	Deli broccoli salads and coleslaw	*Listeria monocytogenes,* Mann2017	King Soopers
10/21/2017	Meijer	Various packaged produce items	*Listeria monocytogenes,* Mann2017	Meijer
10/20/2017	Whole Foods	Salads sold by the pound at salad bar	*Listeria monocytogenes*	Whole Foods Market
10/20/2017	Albertsons, Safeway Vons, Pak'N Save	Fresh vegetable trays and cups	*Listeria monocytogenes,* Mann2017	Albertsons, Safeway Vons, and Pak'N Save

Date	Brand Name(s)	Product Description	Recall Reason	Company
10/19/2017	Mann's	Vegetable Products	*Listeria monocytogenes,* Mann2017	Mann Packing
9/2/2017	Southeastern Grocers, Publix Supermarkets	Fajita blend, Stir fry vegetable, Vegetable kabob and more	*Listeria monocytogenes*	Country Fresh Orlando, LLC.
8/23/2017	Great Value	Frozen organic dark sweet pitted cherry products	*Listeria monocytogenes*	SunOpta Inc's subsidiary, etc.
6/8/2017	Goodseed	Soybean sprouts	*Listeria monocytogenes*	Happy Sprout Inc
6/8/2017	CC Kitchens	Salad and Slaw kits containing leafy greens	*Listeria*	CC Kitchens
6/8/2017	Kroger, Club Chef LLC	Snack kits containing vegetables	*Listeria*	Club Chef LLC
5/12/2017	Peak; Harris Teeter Farmer's Market	Spinach	*Listeria monocytogenes*	The Horton Fruit Co., Inc.
4/11/2017	Season's Choice	Sweet Peas	*Listeria monocytogenes*	Lakeside Foods, Inc.
4/4/2017	Season's Choice	Frozen Peas	*Listeria monocytogenes*	Lakeside Foods
3/16/2017	(no brand name) Edamame	Edamame (Soybeans)	*Listeria monocytogenes*	Advanced Fresh Concepts Franchise Corp.
2/15/2017	Veggie Noodle Co	Butternut Spirals	*Listeria monocytogenes*	Veggie Noodle Co
2/1/2017	Sunmba	Frozen Ajiaco (vegetables mix)	*Listeria monocytogenes*	Barberi International Inc.
9/15/2016	Ossie's	Ready to eat salads	*Listeria monocytogenes*	SM Fish Corp
8/26/2016	Bi-Lo, Harris Teeter, Fresh Point, others	Fresh-cut vegetable products	*Listeria monocytogenes*	Country Fresh, LLC
8/19/2016	Laura Lynn, Key Food, Better Value	Frozen cut corn	*Listeria monocytogenes*	Cambridge Farms, LLC
7/28/2016	Watts Brothers Farms, Tader Joe's	Organic green peas, mixed vegetables and super sweet corn	*Listeria monocytogenes*	ConAgra
7/22/2016	IQF	Cut green beans	*Listeria monocytogenes*	JML Ingredients, Inc.
6/21/2016	C&W	Early harvest petite peas and petite peas	*Listeria monocytogenes*	Pinnacle Foods, Inc.
6/20/2016	Bountiful Havest, First Street, Great Value, others	Frozen green peas and frozen mixed vegetables	*Listeria monocytogenes*	National Frozen Foods Corporation
6/16/2016	HelloFresh	Frozen Peas	*Listeria monocytogenes*	HelloFresh
6/2/2016	Bybee's, Columbia River Organics, others	Frozen Organic and traditional fruits and vegetables	*Listeria monocytogenes*, CRF2016	CRF Frozen Foods
5/21/2016	Albertsons-Safeway, others	Oriental Salad with ginger dressing	*Listeria monocytogenes*	Papa John's and Produce, Inc.

Date	Brand Name(s)	Product Description	Recall Reason	Company
5/17/2016	Tai Pei, Trader Joe's, Hy-Vee, more	Frozen foods	*Listeria monocytogenes*, CRF2016	Ajinomoto Windsor, Inc.
5/16/2016	Stahlbush	IQF green beans	*Listeria monocytogenes*	Stahlbush Island Farms, Inc.
5/12/2016	Piggly Wiggly	Yellow cut corn	*Listeria monocytogenes*, CRF2016	McCall Farms, Inc.
5/10/2016	Simple Truth	Organic mixed vegetables	*Listeria monocytogenes*, CRF2016	The Kroger Co.
5/9/2016	Natural Directions	Organic mixed vegetables and orgnic green peas	*Listeria monocytogenes*, CRF2016	NORPAC Foods, Inc.
5/9/2016	Pictsweet	Frozen vegetables	*Listeria monocytogenes*, CRF2016	The Pictsweet Company
5/7/2016	Kroger, P$$T	Frozen vegetables	*Listeria monocytogenes*, CRF2016	The Pictsweet Company
5/6/2016	Central Market Organic, others	Vegetable products containing organic peas	*Listeria monocytogenes*, CRF2016	Twin City Foods, Inc.
5/6/2016	Harris Teeter	Frozen organic corn and frozen vegetables	*Listeria monocytogenes*, CRF2016	Harris Teeter
5/5/2016	Pita Pal	Salads	*Listeria monocytogenes*, CRF2016	Pita Pal Foods, LP
5/5/2016	Watts Brothers Farms, Trader Joe's	Frozen vegetable products	*Listeria monocytogenes*, CRF2016	ConAgra
5/2/2016	Bybee's, Columbia Rivers Organics, others	Frozen organic and traditional fruits and vegetables	*Listeria monocytogenes*, CRF2016	CRF Frozen Foods
4/23/2016	True Goodness, Wellsley Farms, others	Frozen vegetable products	*Listeria monocytogenes*, CRF2016	CRF Frozen Foods
4/5/2016	HEB Ready Fresh Go	Cut fruit packages containing apples	*Listeria monocytogenes*	Fresh from Texas
4/1/2016	Wylwood	Fresh frozen broccoli	*Listeria monocytogenes*	Alimentos Congelados
3/3/2016	BI-LO, LLC	Cantaloupes	*Listeria monocytogenes*	BI-LO, LLC
1/22/2016	Dole, Fresh Selections, others	Packaged salad	*Listeria monocytogenes*	Dole Fresh Vegetables, Inc.

[1]Data from U.S. Food & Drug Administration, Recalls, Market Withdrawals, & Safety Alerts, filtered for *Listeria* and produce related recalls only from 2016 to 2019.

[2]All produce-related recalls in the United States from *Listeria* spp. may not be captured in this table.

1.6 *Listeria* spp. prevalence in produce associated environments

Listeria spp. have been known to be pervasive in natural environments and are commonly found in soil, water, and in decaying organic matter (Dowe et al., 1997; Linke et al., 2014; Weis and Seeliger, 1975; Welshimer, 1960). *Listeria* spp. have also been frequently recovered from livestock feed, sewage, and animals (Armstrong, 1985, Petran et al., 1988), and are a recognized contaminant in pre-harvest and post-harvest environments of a variety of crops. For example*, L. monocytogenes* was recovered from 30% of water samples collected from irrigation and non-irrigation water sources across 21 produce farms in New York State, as well as from soil samples collected in fields associated with these farms (Strawn et al., 2013). Contamination with *L. monocytogenes* on produce primary production farms (i.e. in fields prior to being harvested) has also been observed, though prevalence is typically low (Fenlon et al., 1996; Ferreira et al., 2014).

L. monocytogenes can also be regularly recovered from produce in retail environments and surfaces in these environments that come into contact with produce. A study as far back as 1987 investigated the presence of *Listeria* spp. in 10 types of fresh produce obtained from super markets in U.S. (Heisick et al., 1989). They recovered four species including *L. monocytogenes*, *L. innocua*, *L. seeligeri* and *L. welshimeri*. In total, 48% of the isolates were identified as *L. monocytogenes* (Heisick et al., 1989). A review from 1990s by Beuchat (1996) examined the pathogenic microorganisms contamination in fresh produce with studies reporting contamination of a variety of raw vegetables including; bean sprouts, cabbage, cucumbers, leafy vegetables, potatoes, prepacked salads, radish, salad vegetables, and tomatoes (Beuchat, 1996). Other studies have looked at more diverse produce products in retail establishments. A cross-sectional sampling of 121 retail

establishments looking at RTE deli salads found *L. monocytogenes* in 7.7% of intact products and in 4.8% of processed/sliced products (Sauders et al., 2009). This study also recovered *L. monocytogenes* from 12.5% of produce preparation areas and 25% of produce area floor drains (Saders et al., 2009).

1.7 Transient and persistent strains

Outbreaks and investigations have demonstrated that *L. monocytogenes* can contaminate food as a transient organism in a food processing environment or it can establish itself as a persistent resident (Kathariou, 2002). Since *L. monocytogenes* is ubiquitous in the natural environment, transient contamination of a facility would mean a strain was introduced from the outside environment, but it would most likely be eliminated in the facility by cleaning and sanitation (Hoelzer et al., 2011). The presence of these transient strains of *L. monocytogenes* in food industry is inevitable, especially in produce PHP facilities (Strawn et al., 2013). The higher priority goal for food manufacturing facilities is to prevent pathogens from becoming permanent residents. Many studies have demonstrated that *L. monocytogenes* is capable of becoming established in food manufacturing environments. Once a persistent strain becomes established in a facility it may remain a dormant member of the facility microbiome for years. In 1990, a *L. monocytogenes* strain was found in several locations in an ice cream facility (Miettinen et al., 1999). This strain was isolated from the production area over the next seven years (Miettinen et al., 1999). Similarly, a deli meat production facility was found to have a persistent strain of *L. monocytogenes* serotype 4b, detected over a period of 12 years (Tompkin, 2002; Wenger et al., 1990). This strain also matched a strain that was isolated

from a human patient with listeriosis. Persistence of *L. monocytogenes* in produce PHP facilities has not been the focus of previous investigations.

1.8 Environmental monitoring: guidance and control of *Listeria* spp.

Food processing facilities that produce RTE foods with environmental exposure are expected to implement effective sanitation controls to mitigate the risk of *L. monocytogenes* contamination. The primary verification mechanism to demonstrate the effectiveness of sanitation controls is through pathogen environmental monitoring programs (PEMPs). A strategic PEMP is designed to detect harborage points and growth niches of *L. monocytogenes* (Ferreira et al., 2014). Many resources have been developed to help food manufacturing facilities control *Listeria monocytogenes* in production environments. Two most recent guidance documents that address *L. monocytogenes* in the produce industry include the Food and Drug Administration's (FDA) "Control of *Listeria monocytogenes* in Ready-To-Eat (RTE) Foods: Guidance for the Industry" and the 2018 "Guidance on Environmental Monitoring and Control of *Listeria* for the Fresh Produce Industry" developed by the United Fresh Food Safety & Technology Council. Both of these documents provide detail on control of *L. monocytogenes* in foods, and describe best practices spanning production operations to packaging (i.e. best agricultural practices and listericidal treatments of the product). Both documents also have sections on steps and procedures for environmental monitoring.

In the FDA's guidance the main goals of environmental monitoring for *L. monocytogenes* are to (i) verify the effectiveness of control programs for *L. monocytogenes* (cleaning and sanitation) and (ii) create a program that helps to seek and

destroy *L. monocytogenes* (FDA, 2017). The guidance suggests using a zoning approach to sampling (i.e. sampling in zones 1-4), ensuring there are detailed written procedures for environmental monitoring programs, and collecting and testing samples from food contact (FCS) and non-food contact (NFCS) surfaces (FDA, 2017). At least five samples should be taken from FCS (zone 1) and five from NFCS (zones 2-4) (FDA, 2017). A detailed corrective action plan is also critical when a sample has tested positive for *Listeria* spp. or *Listeria monocytogenes*. It is extremely important for a facility to respond to positives, especially when found on FCS. If a FCS is consistently positive (3 positives in a row) for *Listeria* spp. or if the product is consistently positive for *L. monocytogenes*, the product may need to be reprocessed, destroyed and a recall should be considered (FDA, 2017).

The United Fresh *Listeria* guidance includes goals and suggestions similar to FDA's *Listeria* Guidance with other useful information specifically related to the produce industry. This includes guidance on where to sample and not to sample when looking for *L. monocytogenes*. For example, this guidance reiterates that not all produce facilities are equally vulnerable to *L. monocytogenes* contamination and a risk assessment should be conducted before environmental monitoring begins (Bierschwale et al., 2013). Facilities that are dry packinghouses, that do not have equipment or conveyors that are washed or wet, or if they work with products that are rarely consumed raw may not need a strict environmental monitoring regime (Bierschwale et al., 2013). This guidance also highlights that it is important to detect *Listeria* spp. in initial raw product rooms or high traffic entry points before it can spread to the rest of the facility and become a resident strain (Bierschwale et al., 2013).

In conclusion, there are many resources available for PHP facilities that focus solely on *Listeria* control and give details on how to construct PEMPs. These resources are in response to industry-wide confusion over FSMA requirements and government guidance documents, as well as confusion and misinterpretations of the overall goal of PEMPs. All facilities are different and require different levels of environmental monitoring and attention to food safety. Overall, each individual facility (even if a part of a large company) needs to determine their level of risk for *Listeria* spp. contamination, create a specific environmental monitoring program for their facility, and establish corrective actions in the event of a positive result.

1.9 Summary of research approach

The increased reporting of foodborne outbreaks linked to produce has led to increased federal oversight of the U.S. and global food supply. The unique properties of *L. monocytogenes* support its ability to survive and persist in diverse environments in the produce industry. To better guide long-term strategies to control and mitigate the contamination of *L. monocytogenes* in produce, foundational observational studies are needed to determine the prevalence and diversity of *Listeria* in PHP facilities. The research presented in this thesis was designed to begin building this foundation of knowledge in the Pacific Northwest (PNW). Research objectives and a summary of major tasks are listed below:

Objective 1: Characterize produce handling and packing (PHP) facilities in PNW in terms of operational characteristics as well as *Listeria* prevalence (Chapter 2).

Major Task 1: Conduct a survey of PHP facilities to describe current sanitation protocols and environmental monitoring strategies related to *Listeria* control.

Major Task 2: Collect and analyze environmental samples from seven PHP facilities (e.g., vegetable and fruit manufacturing, packinghouses, distribution/holding). A total of 350 environmental samples (50/facility) were collected and tested for *Listeria* spp. on non-food contact surfaces (NFCS).

Major Task 3: Conduct investigative environmental sampling at a PHP facility with high prevalence of *Listeria* spp. to identify high risk contamination and harborage sites, and potential for cross-contamination. An additional 100 NFCS environmental samples were collected and analyzed for the presence of *Listeria* spp.

Objective 2: Explain the diversity of *Listeria* spp. isolated from PHP facilities to indicate the potential for persistence, virulence, and development of resistance (Chapter 3).

Main Task 1: Use multiplex PCR assay to determine serogroups of all *L. monocytogenes* isolates (n = 108).

Main Task 2: Determine the susceptibility of *Listeria* spp. isolates (n = 165) to 18 antibiotics.

Objective 3: Support the PNW specialty crop industries in their efforts to mitigate *Listeria* spp. contamination through educational workshops.

Main Task 1: Design pathogen environmental monitoring program (PEMP) workshop using FDA's guidance document: Control of *Listeria monocytogenes* in Ready-To-Eat (RTE) Foods.

Main Task 2: Deliver workshop to food safety professionals in the PNW to improve understanding of sources of *Listeria* spp. contamination, building a functional environmental monitoring program for *Listeria* spp., providing hands-on training for environmental sample collection, and facilitating decision-making for positive environmental samples.

Chapter 2: Prevalence of *Listeria* spp. in Produce Handling and Processing Facilities in the Pacific Northwest

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2.1 Abstract

To help mitigate outbreaks and recalls due to *L. monocytogenes,* regulations associated with the Food Safety Modernization Act emphasize controlling *Listeria* spp. in food handling and processing environments. Studies have investigated prevalence of *Listeria* in meat, dairy, and seafood operations; however, limited data are available for the produce industry. The objective of this study was to characterize the prevalence of *Listeria* spp. and *L. monocytogenes* in seven produce handling and processing (PHP) facilities in the Pacific Northwest. PHP facilities are defined as facilities that handle, pack, wash, or process raw agricultural commodities in various steps throughout the food chain prior to being sold in the retail sector. Environmental swabs were collected in high-risk areas (near raw product entry points) on two occasions (n = 50/facility). The presence of *Listeria* spp. in the samples was confirmed using a modified ISO 11290-1 method, followed by speciation using Microgen® *Listeria*-ID. *Listeria* spp. were found in 5/7 facilities, with *L. monocytogenes* recovered in all positive facilities. Due to the high prevalence (26%) of *Listeria* spp. in Facility A, two additional sampling rounds (n = 50/round) were conducted. In total, 44/150 (29.3%) environmental swabs contained at least one *Listeria* spp. This study demonstrated the high prevalence of *Listeria* spp. near raw product entry points in diverse PHP facilities. Drains, entry areas, and portable equipment were surfaces that consistently tested positive for *Listeria* spp. during active production.

Keywords: Environmental monitoring**,** Produce**,** *Listeria***,** *Listeria monocytogenes,* Prevalence**,** Packinghouse

2.2 Introduction

The United States (U.S.) public health agenda includes a decrease in the annual number of foodborne illnesses including a targeted reduction of listeriosis. The Food Safety Modernization Act (FSMA) was passed in 2011 with the intention of supporting this public health goal. Due to the high morbidity and mortality rate of listeriosis, FSMA regulations, especially the Preventive Controls for Human Food (PCHF) rule, include emphasis on controlling *L. monocytogenes* in food processing facilities where ready-to-eat products are openly exposed to the environment. In 2017, the U.S. Food and Drug Administration (FDA) issued a new draft guidance document, addressing best practices and control of *L. monocytogenes* in ready-to-eat foods (FDA, 2017).

The PCHF and guidance documents provide a strategy for mitigating environmental pathogens in food processing facilities; however, these do not apply to farm and mixed-type facility operations that handle fresh produce. Instead, these facilities are subject to the Produce Safety Rule (PSR). These facilities face different challenges, as they often receive produce directly after harvest and quickly handle it to facilitate refrigerated storage or distribution without an antimicrobial intervention (i.e., kill step). There are minimal expectations or support for these facilities to mitigate the potential for *L. monocytogenes* contamination, despite significant listeriosis outbreaks being linked to produce farm operations. One of the most significant listeriosis outbreaks, leading to 33 deaths, was linked to contaminated cantaloupe from a single farm operation (CDC, 2012).
The unhygienic production environment was likely the reason for the contamination of the whole cantaloupes (McCollum et al., 2013). In 2015, a multistate listeriosis outbreak associated with caramel apples hospitalized 34, with 7 deaths reported (CDC, 2015).

Environmental samples from an apple production facility in California recovered *L. monocytogenes* strains that were indistinguishable from strains associated with this outbreak (Angelo et al., 2017). Most recently, there have been multistate outbreaks of listeriosis linked to frozen vegetables and another linked to packaged salads, both in 2016 (CDC, 2016b, 2016a).

Listeria spp. are saprophytes that are commonly present in the soil of various agricultural landscapes (Chapin et al., 2014; Linke et al., 2014; Strawn et al., 2013; Vivant et al., 2013). Therefore, it is expected that freshly harvested produce, particularly crops that are close proximity to the topsoil, would be at risk for *Listeria* spp. contamination. As these crops are harvested and transported into the packing or processing facility, they carry *Listeria* cells that have the potential to establish themselves in niches and lead to chronic contamination of subsequently processed produce. Few studies have investigated the prevalence of *Listeria* spp. and *L. monocytogenes* in produce and produce handling and processing (PHP) environments (Gianfranceschi et al., 2002; Hoelzer et al., 2011; Leong et al., 2014, 2017; Prazak et al., 2002; Sauders et al., 2009; Tan et al., 2019). These studies demonstrated *Listeria* spp. prevalence in specific produce associated environments and regularly combined results from product testing, retail, and processing environments. Data are lacking for the environmental prevalence of *Listeria* spp. in specific U.S. regions (e.g., Pacific Northwest, [PNW]) strictly related to the PHP industry.

Annually in the US, there are 15 to 30 recalls of produce products due to risk of contamination with *L. monocytogenes* (FDA, 2019). These recalls come at a significant cost to the produce industry, both a direct financial cost as well as future lost revenue due to waning consumer confidence. The 2006 *Escherichia coli* O157:H7 spinach outbreak,

sickening more than 200 and killing 3 people (CDC, 2006), was followed with dramatic economic loss and burden. The FDA warned about all spinach grown in the U.S. essentially shutting down the industry for several months (Arnade et al., 2009). It was estimated that U.S. spinach growers lost nearly $12 million while the retail sector loss was over $63 million (Ribera et al., 2012). The produce industry has limited data to support risk management and mitigation strategies for *L. monocytogenes*. Prevalence data specific to the produce industry, and especially in facilities covered by different regulations, as well as regional differences, would be helpful to inform future food safety strategies. The overall objective of this study was to estimate the prevalence of *Listeria* spp. in PHP facilities in the PNW states of Oregon and Washington. A secondary objective was to identify potential routes of *Listeria* spp. movement in PHP facilities.

2.3 Materials and methods

2.3.1 Produce handling and processing facilities

A flyer (Appendix A) about environmental monitoring research was distributed to regional PHP facilities at workshops and through extension listservs. No financial incentive was offered to encourage participation. Facilities that responded to the participation request were contacted via email to verify eligibility and provide additional information on the research goals, expectations associated with participation, and anonymity. For the purpose of this research, the definition of a PHP facility was one that grew, harvested, packed, and/or processed produce in Oregon or Washington state. Additional phone calls between researchers and PHP facility personnel were completed to gather relevant information about their operation, to schedule site visits, sample collection dates, and to

gather other relevant information. Relevant information included number and type of crops in facility, production details (i.e. handle, pack, process produce or combination), final products, sanitation practices, and environmental monitoring history (Question List in Appendix B). Seven PHP facilities agreed to participate in the study; relevant information about each facility is summarized in Table 2.1.

2.3.2 Environmental sample collection

Each participating PHP facility was visited to identify and categorize environmental sampling sites to maintain consistency across facilities. Final sampling site selections and number of samples per sampling round are shown in Table 2.2. A template of the sample collection form can be found in Appendix C. Oregon State University laboratory staff collected all environmental samples from each facility within 2-3 h of the beginning of the production day. Exact sampling locations for each facility were documented on a facility map with a brief description, along with a digital photograph. A supervisor from each facility was present and actively observed environmental sample collection. A copy of all written information and metadata were provided to the facility prior to leaving the property. Each facility was visited for sample collection at least twice (Round A and Round B) during the 2018-2019 processing year. Findings from these initial rounds informed decisions for future site visits and additional swabbing rounds (Rounds C and D). Environmental samples were collected by swabbing an approximate area of 930 cm^2 (30.5 cm x 30.5 cm) with a sponge-stick moistened with neutralizing buffer (3M, St. Paul, MN, USA) on non-food contact surfaces (NFCS) only. The area was swabbed five times vertically with one side, and five times horizontally with one side.

Table 2.1. Produce handling and processing (PHP) facilities that participated in this study.

Facility	# of Commodities	Kill Step	FSMA Compliance Type[a]	# of Employees	Production (Seasonal or year-round)	Cleaning and Sanitation Frequency	Sanitizers[b]	Environmental Monitoring Program
A	48[c]	No	PSR only	30-50	Year-round	Daily	PAA QAC	No
C	3	Yes	PC only	80-150	Year-round	Daily	ClO^- QAC	Yes; 10/month; *Listeria* and *Salmonella*
D	3	No	PC only	25-100	Year-round	Daily	Not disclosed	Yes; no additional details.
E	3	No	PSR & PC	25-100	Seasonal	Daily	ClO^-	Yes; Daily, varies on # of samples; *Salmonella* spp. *E. coli* (generic), *Listeria* spp.
F	26	Yes	PSR & PC	350-400	Year-round	Daily	ClO^-	Yes; 16/week; *Listeria* spp. (if positive will speciate to *L. monocytogenes*), *Salmonella* spp., *E. coli* (generic, check if *E. coli* 0157:H7 if positive)
G	7	No	PSR & PC	25-100	Seasonal	Daily	Not disclosed	Yes; no additional details.
H	3	No	PSR & PC	25-400	Seasonal	Daily	QAC	Yes; *Listeria* spp., a few times per year in drains only

[a]PSR indicates facility is covered under 21 CFR Parts 11, 16, and 112 Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption Rule (Produce Safety Rule) or PC indicates facility is covered under 21 CFR Part 117 Current Good Manufacturing Practice, Hazard Analysis, and Risk-Based Preventive Controls for Human Food Rule (Preventive Controls), or both (PSR & PC).
[b]ClO^- = Hypochlorite; PAA = Peroxyacetic Acid; QAC = Quaternary Ammonium Compound
[c]Facility A handles, washes, and packs most of these commodities; however, some are received packaged and simply stored prior to distribution

Table 2.2. Environmental samples from each sampling round and sample site description.

Sample Site	Sampling Rounds[1]			
	A	**B**	**C**	**D**
Drain	10	10	12-14	12-14
Forklift tire[2]	2	2	4	4
Forklift traffic area (floor)	1	1	4	4
Side of conveyor	1	2	5-7	5-7
Pallet or bin	2	2	2	2
Entry point	0	3	4	4
Portable items	0	5	5	5
Floor below initial production[3]	0	1	1	1
Outside Surface	0	0	3	3
Coolers	0	0	4	4
Other[4]	4	4	4	4
Total	**20**	**30**	**50**	**50**

[1]All facilities were sampled twice (Rounds A and B). A single facility (Facility A) was sampled on two additional visits (Rounds C and D).
[2]Front left tire.
[3]Samples taken at this site were on the floor below where raw product first started production.
[4]Four sample sites for each round were chosen by facility personnel. Examples include hand wash sink drain pipes, walls, temporary equipment, equipment outside, raw product trailers, additional drain and floor locations, waste bins, hollow cracks and crevices on equipment, and concrete seams on floors.

Sampling locations with insufficient surface area (i.e., equipment legs) were swabbed as completely as possible. Swabs were returned to the original bag and held in a cooler at approximately 4°C until all samples had been collected. Samples were transported to the

Food Safety Laboratory at Oregon State University's Food Innovation Center and stored at 4°C for up to 48 h prior to analysis.

2.3.3 Analysis of environmental samples for Listeria *spp.*

Demi-Fraser broth (45 ml; DFB, Neogen, Lansing, MI, USA) was added to each environmental sample bag. Sample swabs were massaged by hand or with a stomacher to facilitate the release of bacteria into solution. These pre-enrichments were incubated at 30°C for 24 h. Following incubation, samples were mixed and 100 µl was transferred to 10 ml of Fraser broth (FB, Neogen, Lansing, MI, USA) and incubated at 35°C for 24-48 h with shaking at 200 rpm. Aliquots (10 µl) of the incubated DFB and/or FB were spread plated onto both a Harlequin *Listeria* agar according to the formulation of Ottaviani and Agosti (ALOA, Neogen, Lansing, MI, USA), and PALCAM agar (Neogen, Lansing, MI, USA) and incubated at 35°C for 24-48 h. Following incubation, ALOA and PALCAM plates were evaluated for the presence of typical *Listeria* spp. colonies. *Listeria* spp. colonies appear blue to green on ALOA, with *L. monocytogenes* differentiated by the presence of an opaque halo. *Listeria* spp. develop grey-green colonies with a black precipitate on PALCAM, with no differentiation between species. For each sample, up to 20 colonies displaying morphology typical for *Listeria* spp. were selected and transferred by stabbing to trypticase soy agar (TSA) + 5% defibrinated horse blood (HBA; Hardy Diagnostics, Santa Maria, CA, USA). Hemolysis was evaluated after incubation at 35°C for 24 h and 2-3 colonies were streaked for isolation on TSA + 5% sheep blood (BAP; Hardy Diagnostics, Santa Maria, CA, USA). Isolates were speciated using the Microgen® *Listeria*-ID system (Microgen; Microgen Bioproducts, Camberly, UK) following manufacturer's instructions. Isolates confirmed as

Listeria spp. were suspended in 50% glycerol and stored at -80°C. Isolates were further characterized by Gram-stain reaction, catalase, motility, serogroup, and antimicrobial resistance profiling (Jorgensen et al., 2019a). *Listeria monocytogenes* ATCC 19115 and *L. innocua* ATCC 33090 served as reference strains for morphology and test interpretation. All strains from positive sampling sites (up to three from each positive sampling site) were further evaluated for antimicrobial resistance (AMR) and *L. monocytogenes* strains were serogrouped (Jorgensen et al., 2019a).

2.3.4 Statistical analysis

Significant differences in the prevalence of *Listeria* spp. between facilities were determined using chi-square test of independence using R base package (v. 3.6.0; R Core Team, 2019).

2.4 Results

2.4.1 Prevalence of Listeria *spp. in PNW PHP facilities*

Following two rounds of sampling, *Listeria* spp. were isolated from environmental samples at 5/7 (71%) PHP facilities (Table 2.3). Overall, 15 sampling sites were positive for *L. monocytogenes* and 11 were positive for other *Listeria* spp. (*L. innocua, L. ivanovii,* and *L. welshimeri*; Table 2.3). Sample locations that were positive for *Listeria* spp. included drains, entry points, equipment legs, floors, forklift tires, forklift traffic areas, and other locations (e.g., hand wash sink drain pipes, walls, temporary equipment, equipment outside, waste bins, raw product trailers, additional drain and floor locations, concrete seams on floors). *L. monocytogenes* was the most common species found in PHP facilities

(15/350; 4.3%), followed by *L. welshimeri* (5/350; 1.4%), *L. innocua* (5/350; 1.4%), and *L. ivanovii* (1/350; 0.3%).

Table 2.3. Detection of *Listeria* spp. in environmental sampling sites at PHP facilities during initial rounds of testing (rounds A and B).

Facility	Samples Positive for *Listeria* spp. (%)	Recovered Species (n)	Positive Sample Site Types
A	13/50[1] (26%)	*L. innocua* (3) *L. ivanovii* (1) *L. monocytogenes* (6) *L. welshimeri* (5)	Drain, Entry point, Floor, Other
C	5/50 (10%)	*L. monocytogenes* (5)	Drain, Entry point, Forklift tire, Forklift traffic area
D	1/50 (2%)	*L. monocytogenes* (1)	Drain
E	3/50 (6%)	*L. innocua* (2) *L. monocytogenes* (1)	Drain, Equipment Leg
F	0/50 (0%)	None detected	
G	2/50 (4%)	*L. monocytogenes* (2)	Drain, Entry point
H	0/50 (0%)	None detected	
Total	24/350 (6.8%)	4 species	7 type of sample sites

[1]Two of the samples collected from Facility A were positive for both *L. monocytogenes* and *L. welshimeri*.

Facilities significantly differed in the prevalence of *Listeria* spp. in environmental samples (p <0.0001). *Listeria* spp. was not detected in any of the environmental samples collected from Facilities F and H. Facilities C, D, E, and G had a low percentage (≤10%) of environmental samples that were positive for *Listeria* spp. Facility A had the highest number of environmental samples that were positive for *Listeria* spp. (13/50; 26%); this

facility contributed >50% of the positive samples for the entire study. Facility A was the only facility where *L. welshimeri* and *L. ivanovii* were detected. Due to this high prevalence, Facility A was the focus of subsequent rounds of sampling (rounds C and D). The survey in Table 2.1 reports a few facility characteristics that may be contributing to the highest *Listeria* spp. prevalence observed in Facility A. Notably, this facility washes and packs (no processing or kill-step) nearly 50 different commodities year-round, drastically higher than Facility H that processes three commodities seasonally (6-8 months/year) and has an environmental monitoring program for *Listeria* spp. Facility H had no positives for *Listeria* spp. Additionally, concerns of *Listeria* spp. occurrence in Facility A has only recently become a part of the facilities food safety plan, having no environmental monitoring program. This is also considerably different from Facility F, which had no positives. Facility F tests for *Listeria* spp. weekly (n = 16 samples), with further speciation upon a positive. Though no significant correlations have been made, looking at general facility characteristics provides information that may support the variations observed in *Listeria* spp. prevalence between facilities.

2.4.2 Distribution of Listeria *spp. in Facility A*

Overall results from four rounds (A, B, C, D) of environmental sampling at Facility A are shown in Figure 2.1. Initial sampling rounds (A and B) were focused in the main receiving area where sorting, washing, and packing occur. During the first round of sampling in Facility A (September 2018), only drain samples (3/20; 15%) were positive for *Listeria* spp. *L. innocua* isolates were recovered from a single trench drain (drain #1) at two different locations and *L. monocytogenes* was isolated from a second trench drain (drain

#2). During the second round of sampling (January 2019), the frequency of isolating *Listeria* spp. increased to 10/30 (33%) samples. Sampling sites positive for *Listeria* spp. included drains (n = 5), the floor underneath raw product intake (n = 1), and the floor near facility entry points (n = 1). The remaining positive sampling sites were associated with a raw produce trailer that was driven into the facility to expedite unloading and sorting of raw product. A trailer tire (n = 1) and the floor near the trailer (n = 2) were positive for *Listeria* spp. Four *Listeria* species were isolated from this round of sampling: *L. welshimeri* (n = 5), *L. monocytogenes* (n = 5), *L. innocua* (n = 1) and *L. ivanovii* (n = 1). *L. welshimeri* was recovered from a single trench drain (drain #1) at three different locations, the raw product trailer tire (n = 1), and the floor below the raw product trailer (n = 1). *L. monocytogenes* was recovered from two trench drains (drains #1 and #2) at single locations per drain. *L. monocytogenes* was also recovered from the floor near an entry point, the raw product trailer tire, and the floor surrounding the raw product trailer. *L. innocua* was recovered from the floor surrounding the raw product trailer and *L. ivanovii* was found in a trench drain (drain #2).

Subsequent intensified sampling rounds (n = 50/round) at Facility A expanded sampling into coolers, loading docks, transition areas, and to areas outside the facility. Round C (March 2019) and round D (April 2019) resulted in 16/50 (32%) and 15/50 (30%) samples testing positive for *Listeria* spp., respectively. Positive sampling sites from round C included drains (n = 6), the floor underneath raw product intake (n = 1), fatigue stools used by production employees (portable items, n = 2), a forklift tire (front left tire, n = 1), entry points (n = 2), samples taken on outside surfaces (n = 3) and in the cooler (n = 1). *L. monocytogenes* (n = 5) was recovered from all three drains in the

sorting/washing/packing area (drains #1-3). All three outside samples (outside drain, concrete crack and tractor tire) were positive for *L. monocytogenes.* *L. monocytogenes* was also found in a condensation pool near the entry point of one of the day-use coolers used to store bulk raw produce. *L. welshimeri* was recovered from one drain sample (drain #1), a fatigue stool, and from an entry point. *L. innocua* was isolated from the outside drain. Positive sample sites during the fourth round (round D) of testing included drains (n = 5), fatigue stools (portable items, n = 2), entry points (n = 2), outside sampling locations (n = 5), and the floor condensation in the raw produce storage cooler (n = 1). *L. monocytogenes* (n = 13) and *L. innocua* (n = 4) were the two species recovered during round D. *L. monocytogenes* was found in two drains at multiple locations (drain #1, drain #3), two different fatigue stools, the floor near an entry point, and in multiple outside samples, including the three positive sampling sites from round C.

The cooler floor entry point condensation site was again positive for *L. monocytogenes.* *L. innocua* was recovered from one drain (drain #3), one fatigue stool and the floor near two entry points.

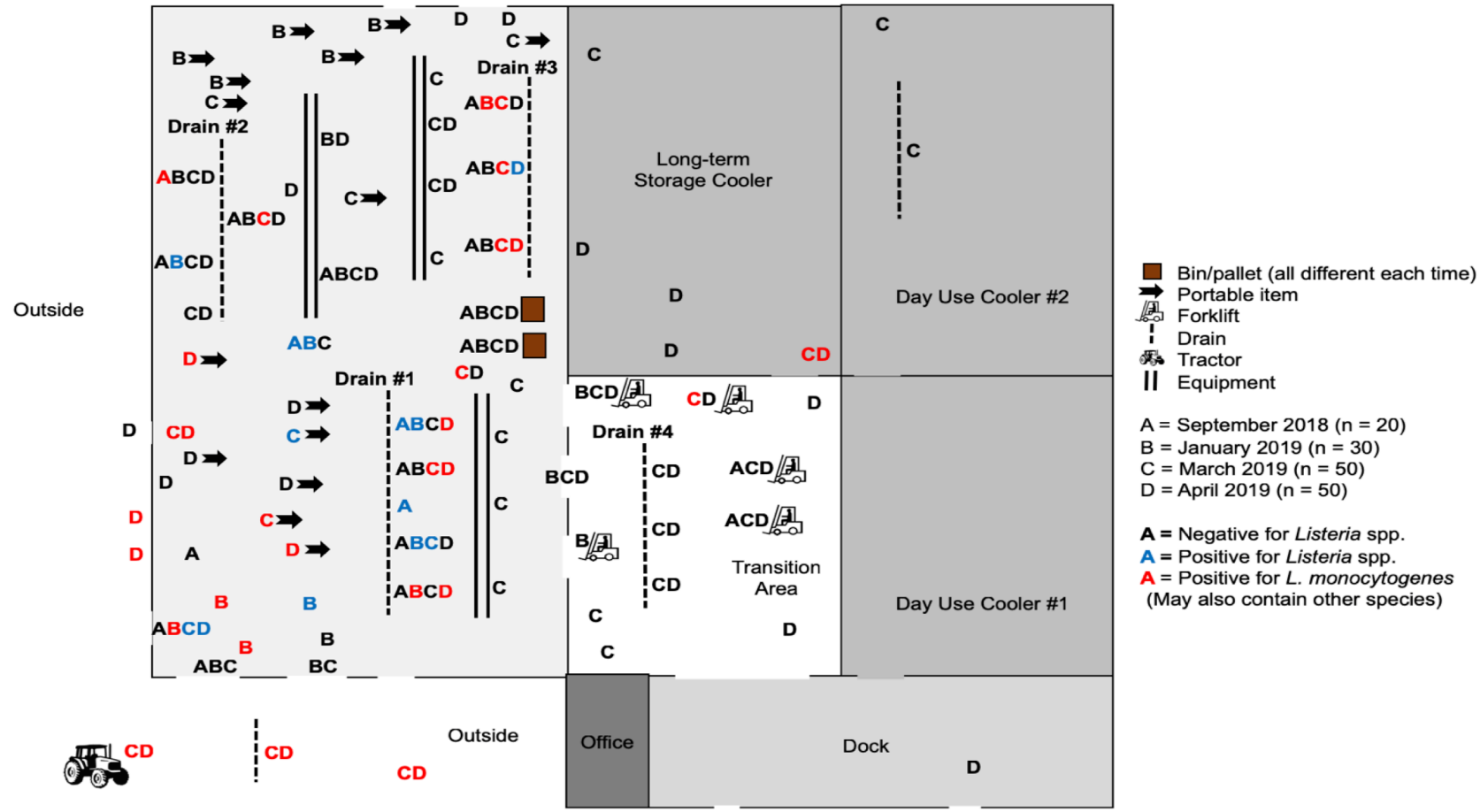

Figure 2.1. Facility A map layout with all sampling rounds identifying positive sites for *Listeria* spp. and *L. monocytogenes*. Samples rounds were done at four different times periods; September 2018 (**A**, n = 20), January 2019 (**B**, n = 30), March 2019 (**C**, n = 50), and April 2019 (**D**, n =50). Negative samples are highlighted in black (**A**, **B**, **C**, **D**), positive samples for *Listeria* spp. are highlighted in blue (**A**, **B**, **C**, **D**) and positive samples for *L. monocytogenes* are highlighted in red (**A**, **B**, **C**, **D**). Other *Listeria* spp. may have also been recovered from red sample sites (positive for *L. monocytogenes*). *Listeria* spp. recovered include *L. innocua*, *L. monocytogenes*, *L. ivanovii* and *L. welshimeri*.

In total, *Listeria* spp. were recovered from 44/150 samples (29%) for all sampling rounds from Facility A. Figure 2.2 shows examples of sampling sites separated into categories based on frequency of sampling site testing positive for *Listeria* spp. in each sampling round: A) consistently positive for *Listeria* spp. (positive all rounds), B) intermittently positive for *Listeria* spp. (positive in at least one round), C) consistently negative for *Listeria* spp. (negative all rounds). Sites that were consistently positive included all drains in the production area (4/4 rounds), an outdoor tractor tire (2/2 rounds), a wet floor location in a cooler near a forklift entry point (2/2 rounds), and production employee fatigue stools (2/2 rounds). A sample taken on wood floors in front of bathrooms (1/1 round) is also included in figure 2.2, though this is not included in our statistical analysis as it was only sampled and positive one round. *Listeria* spp. were commonly recovered from floors near entry points and on the processing floors (3/4 rounds), whereas they were only occasionally found on forklifts (1/4 rounds). *Listeria* spp. were not recovered from the outside of wood bins holding raw products, sides of processing equipment (0/4 rounds), other portable items (0/3 rounds) and the drain located in the transition area (drain #4) (Figure 2.1). Species recovered for all rounds from Facility A included: *L. monocytogenes* (n = 32), *L. welshimeri* (n = 8), *L. innocua* (n = 7), and *L. ivanovii* (n = 1).

A) Consistently *Listeria* spp. positive


Inside Drains


Tractor Tire


Cooler Floor


Bathroom Entry


Stool

B) Intermittently *Listeria* spp. positive


Entry Points


Forklift Tire


Processing Floor

C) Consistently *Listeria* spp. negative


Wood Bins


Processing Equipment


Portable Items

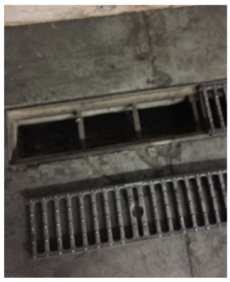
Drain #4

Figure 2.2. Pictures of sampling sites from Facility A categorized by the frequency of detection of *Listeria* spp. across all sampling rounds. Sites were separated into three categories: (A) consistently positive for *Listeria* spp. (positive all rounds); (B) intermittently positive for *Listeria* spp. (positive at least one round); or (C) consistently negative for *Listeria* spp. (negative all rounds). Differences were assessed using chi-square (p = 0.002) and Fisher's Exact (p = 0.00006).

2.4.3 Listeria monocytogenes *movement throughout facility A*

Further characterization of isolates, including serogrouping and AMR profiling (Jorgensen et al., 2019a) suggested that three *L. monocytogenes* strains (identical serogrouping and AMR profile; designated as C_1, D_1, and D_2, Figure 2.3) were isolated from multiple environmental samples. During the third round of testing, three *L. monocytogenes* serogroup 4 isolates with identical AMR profiles (C_1) were found on the production floor, on the floor near an entry point and in drain #3 (Figure 2.3; see photos in Figure 2.2). During produce handling in Facility A, water is in near constant use, and the floor in this area is wet, especially at the entry points and near drains. Movement of employees, forklifts, or portable equipment through standing water or wet surfaces and potential dissemination of *Listeria* spp. throughout the environment is high in this facility. It is likely that this *L. monocytogenes* strain (C_1) entered Facility A from the side door (positive sample on floor near door with heavy foot traffic) and moved to the floor and drain on the bottom of production employee shoes or by forklift tires.

During the fourth round of testing, two sets of *L. monocytogenes* isolates with distinct AMR profiles (D_1 and D_2) were found in Facility A (Figure 2.3). The D_1 strain was isolated from an employee fatigue stool that is moved and stored throughout the facility. Employees stand on these stools throughout the sorting, washing and packing processes and they are stored on the inside perimeter of the facility at various locations. This strain was also isolated from multiple locations in drain #1 and from a single location in drain #3. D_1 may have been tracked into the facility on the bottom of a production employee's shoe and deposited onto the fatigue stool.

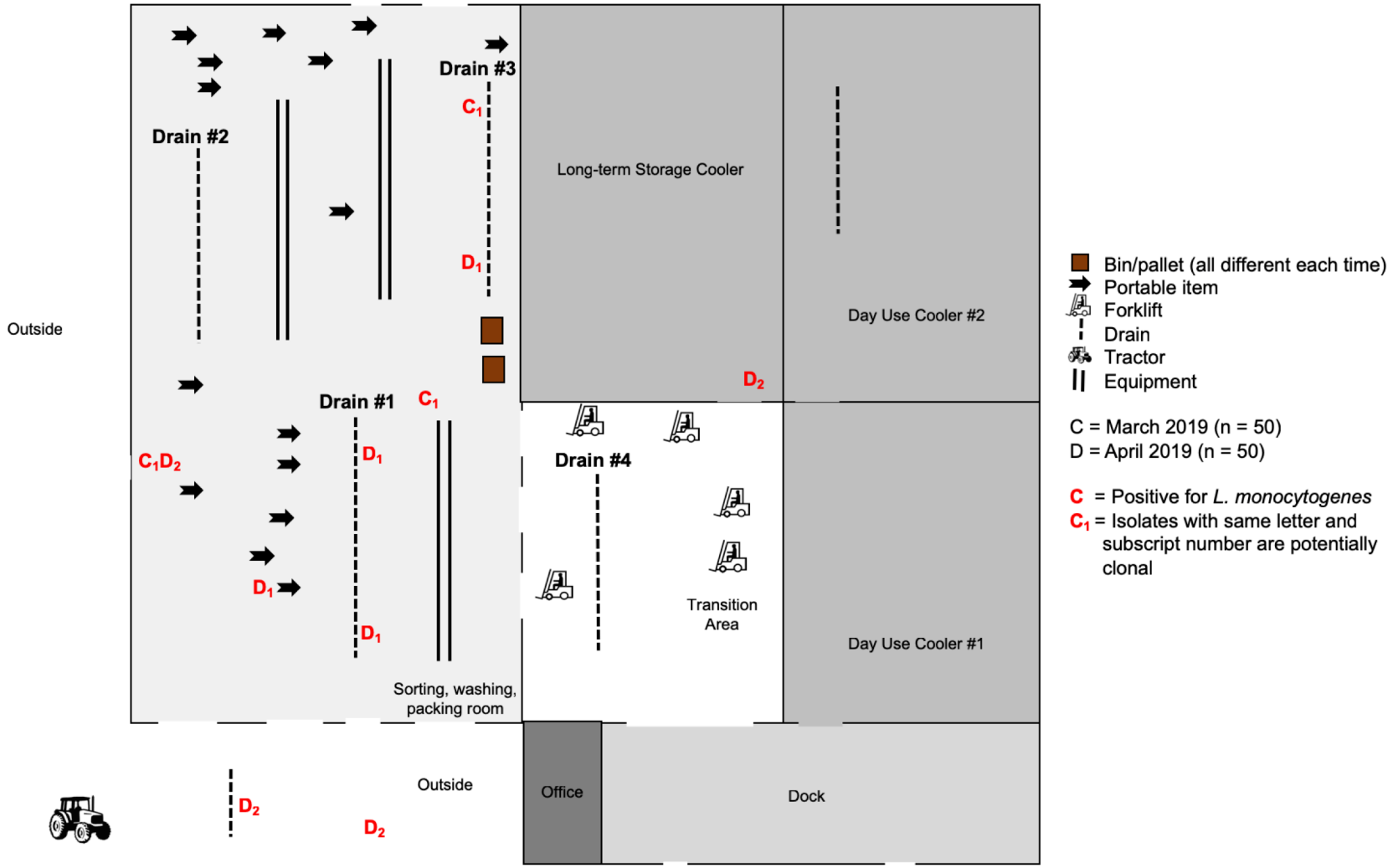

Figure 2.3. Map of *Listeria monocytogenes* isolates suggesting routes of movement within Facility A. Isolates with the same letter and number had the same serogroup and antimicrobial resistance profile, suggesting strong relatedness.

Step stools and floors get washed down and cleaned at the end of production and the strain could then be deposited into the drain. Alternatively, the drain would have been the source for strain D_1. Drains in this facility commonly get clogged with produce waste and overflow onto the floor. Employee traffic in this area could result in *Listeria* spp. transfer to the bottom of worker footwear which would facilitate transfer to the fatigue stools and other areas of the facility.

A third strain (D_2) was isolated from an outside drain and a seam in the concrete staging area in the middle of a produce delivery area at this PHP facility. This strain was also found on the floor by a high-traffic side entry door and on the floor in the corner of a bulk produce cooler that accumulated moisture. This strain may have been brought into the facility by foot traffic or by a forklift and was likely spread to the cooler by extensive forklift traffic. It is possible that bins and pallets could facilitate transfer; however, we did not recovery any *Listeria* spp. on bins or pallets from this facility (0/8).

2.5 Discussion

The prevalence of *Listeria* spp. in PHP facilities in the PNW differed significantly by facility. Facilities fell into three categories, based on *Listeria* spp. prevalence: 1) no *Listeria* spp. detected, 2) low prevalence (≤10% of environmental samples positive), or 3) high prevalence (26% of environmental samples positive). Based on the diversity in facilities and the diversity of activities in PHP facilities, significant differences in *Listeria* spp. prevalence could be expected; however, to date, this is the first study to strategically sample in categorically different facilities under PSR and PCHF rules to demonstrate difference in prevalence and potential contamination routes in these PHP facilities. A

previous three-year study by Leong et al (2017) reported that the prevalence of *L. monocytogenes* varied across facilities from multiple food industries, including five vegetable processing facilities. Certain vegetable processing facilities maintained a prevalence of 0% while one facility had a prevalence of 30% during a single sampling year. Vegetable processing facilities had the highest environmental prevalence for *L. monocytogenes* as compared to the meat, seafood and dairy processing facilities (Leong et al., 2017). A recent study by Tan et al (2019) reported the prevalence of *L. monocytogenes* in three tree fruit processing facilities, with one facility having extremely high prevalence (39/39; 100%)(Tan et al., 2019). There were several factors that this study reported possibly contributing to the high prevalence, including lack of a proper drainage systems, cracks in floors and poor cleaning and sanitation practices (Tan et al., 2019). Additionally, targeted sampling and preliminary environmental data helped researchers from this study readily recover *L. monocytogenes* (Tan et al., 2019).

Facility A had a significantly higher prevalence of *Listeria* spp. in the environment than all other PHP facilities. This facility is an enclosed packinghouse that sorts, cleans, and stores a diversity of crops. Facility A was the only participating PHP facility that was subject to the PSR and not subject to PCHF. The current regulatory landscape for this type of PHP facility does not require, nor encourage, the establishment of a pathogen environmental monitoring program (PEMP); therefore, Facility A had no previous data on the prevalence of *Listeria* spp. in their operation. Based on the results from this study, the food safety personnel (one person) at Facility A began testing environmental samples to verify their cleaning and sanitation programs and they intend to develop PEMP for *Listeria* spp. Close collaboration with the PHP facilities facilitated their ability to

understand parts of their processing environment that may require additional focus for cleaning and sanitation programs.

Sampling locations across PHP facilities were selected to evaluate sites considered to be at high risk for contamination with *Listeria* spp. (i.e., transition floors indoor to outdoor, raw product entry, outside sites and handling areas) (Carpentier and Cerf, 2011; Muhterem-Uyar et al., 2015; Tompkin, 2002). Wood bins and pallets holding raw products were selected for sampling for several reasons. Through preliminary interviews we learned that wood bins and pallets were reused, occasionally transferred and stored outside that facility, and are rarely cleaned and sanitized. The bins and pallets were sampled on the outside, which were always dry. Keeping equipment and the production environment dry is a key component in *Listeria* spp. control (Tompkin, 2002), and could be a contributing factor why we did not see any positives on bins and pallets, rather than the bins being wooden. *L. monocytogenes* was recovered from two wood panels outside the facility (i.e., ground in front of outside bathrooms) that were wet. During the 2017 caramel apple outbreak, *L. monocytogenes* was recovered from the inside of wooden storage bins (Angelo et al., 2017), though it is unknown if the bins were wet or dry. Overall, dry and wet locations across all sites were not included in the metadata, though this data could have contributed to potential positive and negative comparisons.

Other locations, such as sides of processing equipment, were selected to asses potential cross contamination risks to food contact surfaces. These sites are heavily cleaned and sanitized daily and may be a reason why we did not see any positives. Portable equipment (e.g., wheels of scales, wheels of pallet jacks) were selected to investigate cross contamination throughout the facility. The wheels were generally made of hard plastic and

typically wicked off any moisture, and they were consistently dry upon sampling. Drains were targeted based on previously reported high prevalence of *Listeria* spp. in drains in other food production and retail environments (Berrang and Frank, 2012; Hoelzer et al., 2011; Kells and Gilmour, 2004; Tompkin, 2002). At Facility A, 68% (19/28) of drain samples were positive for *Listeria* spp., with 63% (12/19) of drains containing *L. monocytogenes*. Results from our study reaffirm the importance of preventing *Listeria* spp. colonization of drains through scheduled cleaning and sanitization programs. Special care should be taken to prevent drains from being clogged and overflowing onto the floor.

L. monocytogenes was recovered from the floor near the entry way of the bulk produce storage cooler sample site (floor with moisture) in rounds C and D (two separate sampling dates) in Facility A. This could point to *L. monocytogenes* persistence and adaptation at this location and a potential niche site for *Listeria* spp. Upon the second positive in the last round, Facility A management proceeded with corrective actions to frequently clean this location and eliminate the buildup of moisture. *L. monocytogenes* is known to survive and even grow in cold environments (Chan and Wiedmann, 2008; Embarek, 1994; Farber et al., 1999; Sheng et al., 2018). Serogrouping and AMR profile data on this isolate suggest traffic patterns as the mechanism for contamination of this site; however, additional genetic characterization is necessary to characterize isolates at the strain level. It is also possible that there is an environmental niche for *L. monocytogenes* in the cooler. Targeted sampling in the cooler area would help to identify the source and movement of these strains in the facility. Only a few contamination scenarios were addressed in this study based on the available strain relatedness data. There is some

evidence from these scenarios for a connection between high traffic areas and the movement of foot traffic and forklift traffic in raw processing areas. Muhterem-Uyar et al. (2015) observed three *L. monocytogenes* contamination scenarios in 12 meat and dairy facilities across Europe that relate to our findings. From 2,242 environmental samples this study found: (i) sporadic contamination associated with raw material reception and hygienic areas; (ii) hotspots in hygienic processing areas; and (iii) widespread contamination throughout the entire facility (Muhterem-Uyar et al., 2015). Particularly, scenario (i) described by this study corroborates our findings, observing that a contamination from the outside environment was due to the lack of hygiene barriers in raw material reception areas (Muhterem-Uyar et al., 2015). No hygienic barriers are present in Facility A and there is consistent exposure to the outside environment. This is a common and unavoidable situation in packinghouse facilities. The current study demonstrated a high prevalence of *Listeria* spp. entering the facility as product is received and moved. Therefore, focused efforts should be on cleaning and sanitation programs that will prevent cross-contamination from crop to crop or from day to day.

Four species of *Listeria* were isolated from Facility A: *L. innocua*, *L. ivanovii*, *L. monocytogenes*, and *L. welshimeri*. These species are the most commonly recovered species from various food and food-associated environments; however, *L. ivanovii* is less frequently isolated (El-Shenawy, 1998; Gebretsadik et al., 2011; Heisick et al., 1989; Kovacevic et al., 2009; Wilson, 1995). Facility A handles a wide variety of fresh produce. On sampling days, parsnips, cabbage, and beets were being actively handled and packed. *L. ivanovii* and *L. welshimeri* were only recovered from environmental samples on the days in which raw cabbage was being actively packed. Previous research by Prazak et al. (2002)

conducted environmental sampling for *Listeria* spp. at six cabbage packing sheds in Texas. They demonstrated that *L. welshimeri* and *L. ivanovii* are commonly associated with cabbage. When Facility A packs cabbage, they bring the product into the facility on large trailers. The trailers stay in the facility until all the product is unloaded by hand, which may take several hours or days. This production event could be responsible for the sudden influx of *L. welshimeri* during rounds B and C (cabbage packing days), followed by lack of *L. welshimeri* in round D (parsnips packing day). *L. ivanovii* was recovered from one sample in Facility A (1/150; <1%) during round B sampling, and it was not recovered from any other facilities in this study.

The diversity of commodities, production environments, cleaning and sanitation practices, and regulatory requirements varied across the seven PHP facilities that participated in this study. Data to indicate the presence of potential hazards, including *Listeria* spp. are invaluable for food facilities to develop effective mitigation strategies. Verification for control of foodborne pathogens should be individualized for each facility. Despite the high prevalence of *Listeria* spp. in Facility A, further isolate characterization did not demonstrate the presence of any persistent *Listeria* strains. Initial serogrouping and AMR profiling of strains concluded that there were rarely situations where the potentially same strain was recovered during subsequent sampling rounds. This suggests that the majority of *Listeria* spp. strains from Facility A are transient, though further genetic characterization is needed to definitely confirm this hypothesis. Sample collection in all facilities occurred during active facility operations. Samples were not collected after cleaning and sanitation. Results from this study do not support the potential persistence of

Listeria spp. strains in Facility A or that this facility does not have an effective *Listeria* control strategy.

2.6 Conclusions

L. monocytogenes was isolated from the production environment of all PHP facilities in this study that had *Listeria* spp. Our data indicate an increased risk of environmental contamination for PHP facilities that function as packinghouses and handle multiple types of raw produce. The facility with the highest prevalence of *Listeria* spp. is subject to the PSR only and does not have an environmental monitoring program. Participation in this study clearly demonstrated potential risks in their facility and are guiding strategic improvements in the control and sanitation of high traffic areas, such as entry point floors used by employees working on production lines. A high rate of *Listeria*-positive drains demonstrates a continued need to emphasize management of drain cleaning and sanitation to mitigate their potential to contaminate adjacent areas and spread throughout the facility and indirectly transfer to food contact surfaces. Sampling and testing for *Listeria* spp. in close proximity outside the facility may also indicate potential *Listeria* spp. hotspots or sources, and indicate pathways for *Listeria* spp. to be tracked into the facility. Environmental monitoring trends can be a powerful tool within a PHP facility and throughout an industry contributing to the success of food and consumer safety.

2.7 Acknowledgments

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2.8 References

Angelo, K.M., Conrad, A.R., Saupe, A., Dragoo, H., West, N., Sorenson, A., Barnes, A., Doyle, M., Beal, J., Jackson, K.A., Stroika, S., Tarr, C., Kucerova, Z., Lance, S., Gould, L.H., Wise, M., Jackson, B.R., 2017. Multistate outbreak of *Listeria monocytogenes* infections linked to whole apples used in commercially produced, prepackaged caramel apples: United States, 2014-2015. Epidemiol. Infect. 145. https://doi.org/10.1017/S0950268816003083

Arnade, C., Calvin, L., Kuchler, F., 2009. Consumer response to a food safety shock: the 2006 food-borne illness outbreak of *E. coli* O157: H7 linked to spinach. Rev. Agric. Econ. 31, 734–750. https://doi.org/10.1111/j.1467-9353.2009.01464.x

Berrang, M.E., Frank, J.F., 2012. Generation of airborne *Listeria innocua* from model floor drains. J. Food Prot. 75, 1328-1331. https://doi: 10.4315/0362-028X.JFP-12-021.

Carpentier, B., Cerf, O., 2011. Review — Persistence of *Listeria monocytogenes* in food industry equipment and premises. Int. J. Food Microbiol. 145, 1–8. https://doi.org/10.1016/j.ijfoodmicro.2011.01.005

CDC, 2016a. Multistate outbreak of listeriosis linked to frozen vegetables. https://www.cdc.gov/listeria/outbreaks/frozen-vegetables-05-16/index.html (accessed 29 August 2019).

CDC, 2016b. Multistate outbreak of listeriosis linked to packaged salads produced at Springfield, Ohio Dole processing facility. https://www.cdc.gov/listeria/outbreaks/bagged-salads-01-16/index.html (accessed 29 August 2019).

CDC, 2015. Multistate outbreak of listeriosis linked to commercially produced, prepackaged caramel apples made from Bidart Bros. apples. https://www.cdc.gov/listeria/outbreaks/caramel-apples-12-14/index.html (accessed 29 August 2019).

CDC, 2012. Multistate outbreak of listeriosis linked to whole cantaloupes from Jensen Farms, Colorado. https://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/index.html (accessed 29 August 2019).

CDC, 2006. *E. coli* outbreak from fresh spinach. https://www.cdc.gov/ecoli/2006/spinach-10-2006.html (accessed 29 August 2019).

Chan, Y.C., Wiedmann, M., 2008. Physiology and genetics of *Listeria monocytogenes* survival and growth at cold temperatures. Crit. Rev. Food Sci. Nutr. 49, 237–253. https://doi.org/10.1080/10408390701856272

Chapin, T.K., Nightingale, K.K., Worobo, R.W., Wiedmann, M., Strawn, L.K., 2014. Geographical and meteorological factors associated with isolation of *Listeria* species in New York state produce production and natural environments. J. Food Prot. 77, 1919–1928. https://doi.org/10.4315/0362-028X.JFP-14-132

El-Shenawy, M.A., 1998. Sources of *Listeria* spp. in domestic food processing environment. Int. J. Environ. Health Res. 8, 241–251. https://doi.org/10.1080/09603129873516

Embarek, B., 1994. Presence, detection and growth of *Listeria monocytogenes* in seafoods: a review. Int. J. Food Microbiol. 23, 17–34. https://doi.org/10.1016/0168-1605(94)90219-4

Farber, J.M., Peterkin, P.I., 1999. Incidence and behavior of *Listeria monocytogenes* in meat products. In: Ryser, E.T., Marth, E.H. (Eds.), *Listeria*, Listeriosis and Food Safety, 2nd Edition. Marcel Dekker, New York, pp. 505–564.

FDA, 2019. Recalls, market withdrawals, & safety alerts. https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts (accessed 30 August 2019).

FDA, 2017. Draft guidance for industry: Control of *Listeria monocytogenes* in ready-to-eat foods. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/draft-guidance-industry-control-listeria-monocytogenes-ready-eat-foods (accessed 30 August 2019).

Gebretsadik, S., Kassa, T., Alemayehu, H., Huruy, K., Kebede, N., 2011. Isolation and characterization of *Listeria monocytogenes* and other *Listeria* species in foods of animal origin in Addis Ababa, Ethiopia. J. Infect. Public Health 4, 22–29. https://doi.org/10.1016/j.jiph.2010.10.002

Gianfranceschi, M., Gattuso, A., Tartaro, S., Aureli, P., 2002. Incidence of *Listeria monocytogenes* in food and environmental samples in Italy between 1990 and 1999: serotype distribution in food, environmental and clinical samples. Eur. J. Epidemiol. 18, 1001–1006. https://doi.org/10.1023/a:1025849532417

Heisick, J.E., Wagner, D.E., Nierman, M.L., Peeler, J.T., 1989. *Listeria* spp. found on fresh market produce. Appl. Environ. Microbiol. 55, 1925–1927.

Hoelzer, K., Sauders, B.D., Sanchez, M.D., Olsen, P.T., Pickett, M.M., Mangione, K.J., Rice, D.H., Corby, J., Stich, S., Fortes, E.D., Roof, S.E., Grohn, Y.T., Wiedmann, M., Oliver, H.F., 2011. Prevalence, distribution, and diversity of *Listeria monocytogenes* in retail environments, focusing on small establishments and establishments with a history of failed inspections. J. Food Prot. 74, 1083-1095. https://doi.org/10.4315/0362-028X.JFP-10-567

Jorgensen, J., Waite-Cusic, J., Kovacevic, J. 2019a. Diversity of *Listeria* spp. in produce handling and processing facilities in the Pacific Northwest. Submitted to Food Microbiol.

Kells, J., Gilmour, A., 2004. Incidence of *Listeria monocytogenes* in two milk processing environments, and assessment of *Listeria monocytogenes* blood agar for isolation. Int. J. Food Microbiol. 91, 167–174. https://doi.org/10.1016/S0168-1605(03)00378-7

Kovacevic, J., Bohaychuk, V.M., Barrios, P.R., Gensler, G.E., Rolheiser, D.L., McMullen, L.M., 2009. Evaluation of environmental sampling methods and rapid detection assays for recovery and identification of *Listeria* spp. from meat processing facilities. J. Food Prot. 72, 696–701. https://doi.org/10.4315/0362-028x-72.4.696

Leong, D., Alvarez-Ordóñez, A., Jordan, K., 2014. Monitoring occurrence and persistence of *Listeria monocytogenes* in foods and food processing environments in the Republic of Ireland. Front. Microbiol. 5, 436. https://dx.doi.org/10.3389%2Ffmicb.2014.00436

Leong, D., Nicaogáin, K., Luque-Sastre, L., Mcmanamon, O., Hunt, K., Alvarez-Ordóñez, A., Scollard, J., Schmalenberger, A., Fanning, S., O'byrne, C., Jordan, K., 2017. A 3-year multi-food study of the presence and persistence of *Listeria monocytogenes* in 54 small food businesses in Ireland. Int. J. Food Microbiol. 249, 18-26. https://doi.org/10.1016/j.ijfoodmicro.2017.02.015

Linke, K., Rückerl, I., Brugger, K., Karpiskova, R., Walland, J., Muri-Klinger, S., Tichy, A., Wagner, M., Stessl, B., 2014. Reservoirs of *Listeria* species in three environmental ecosystems. Appl. Environ. Microbiol. 80, 5583–5592. https://doi.org/10.1128/AEM.01018-14

McCollum, J.T., Cronquist, A.B., Silk, B.J., Jackson, K.A., O'Connor, K.A., Cosgrove, S., Gossack, J.P., Parachini, S.S., Jain, N.S., Ettestad, P., Ibraheem, M., Cantu, V., Joshi, M., DuVernoy, T., Fogg, N.W., Gorny, J.R., Mogen, K.M., Spires, C., Teitell, P., Joseph, L.A., Tarr, C.L., Imanishi, M., Neil, K.P., Tauxe, R. V., Mahon, B.E., 2013. Multistate outbreak of listeriosis associated with cantaloupe. N. Engl. J. Med. 369, 944–953. https://doi.org/10.1056/NEJMoa1215837

Muhterem-Uyar, M., Dalmasso, M., Sorin Bolocan, A., Hernandez, M., Kapetanakou, A.E., Kuchta, T., Manios, S.G., Melero, B., Minarovi cov, J., Ioana Nicolau, A., Rovira, J., Skandamis, P.N., Jordan, K., Rodríguez-L azaro, D., Stessl, B., Wagner, M., 2015. Environmental sampling for *Listeria monocytogenes* control in food processing facilities reveals three contamination scenarios. Food Control 51, 94–107. https://doi.org/10.1016/j.foodcont.2014.10.042

Prazak, A.M., Murano, E.A., Mercado, I., Acuff, G.R., 2002. Prevalence of *Listeria monocytogenes* during production and postharvest processing of cabbage. J. Food Prot. 65, 1728-1734. https://doi.org/10.4315/0362-028x-65.11.1728

Ribera, L.A., Palma, M.A., Paggi, M., Knutson, R., Masabni, J.G., Anciso, J., 2012. Economic analysis of food safety compliance costs and foodborne illness outbreaks in the United States. HortTech. 22, 150–156. https://doi.org/10.21273/HORTTECH.22.2.150

Sauders, B.D., Sanchez, M.D., Rice, D.H., Corby, J., Stich, S., Fortes, E.D., Roof, S.E., Wiedmann, M., 2009. Prevalence and molecular diversity of *Listeria monocytogenes* in retail establishments. J. Food Prot. 72, 2337–2349. https://doi.org/10.4315/0362-028X-72.11.2337

Sheng, L., Hanrahan, I., Sun, X., Taylor, M.H., Mendoza, M., Zhu, M.-J., 2018. Survival of *Listeria innocua* on Fuji apples under commercial cold storage with or without low dose continuous ozone gaseous. Food Microbiol. 76, 21–28. https://doi.org/10.1016/j.fm.2018.04.006

Strawn, L.K., Gröhn, Y.T., Warchocki, S., Worobo, R.W., Bihn, E.A., Wiedmann, M., 2013. Risk factors associated with *Salmonella* and *Listeria monocytogenes* contamination of produce fields. Appl. Environ. Microbiol. 79, 7618–7627. https://doi.org/10.1128/AEM.02831-13

Tan, X., Chung, T., Chen, Y., Macarisin, D., LaBorde, L., Kovac, J., 2019. The occurrence of *Listeria monocytogenes* is associated with built environment microbiota in three tree fruit processing facilities. Microbiome 7, 115. https://doi.org/10.1186/s40168-019-0726-2

Tompkin, R.B., 2002. Control of *Listeria monocytogenes* in the food-processing environment. J. Food Prot. 65, 709–725. https://doi.org/10.4315/0362-028X-65.4.709

Vivant, A.-L., Garmyn, D., Piveteau, P., 2013. *Listeria monocytogenes*, a down-to-earth pathogen. Front. Cell. Infect. Microbiol. 3, 87. https://doi.org/10.3389/fcimb.2013.00087

Wilson, I.G., 1995. Occurrence of *Listeria* species in ready to eat foods. Epidemiol. Infect. 115, 519–26. https://doi.org/10.1017/s0950268800058684

Chapter 3: Diversity of *Listeria* spp. in Produce Handling and Processing Facilities in the Pacific Northwest

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3.1 Abstract

Several recent outbreaks of *Listeria monocytogenes* have been linked to fresh and frozen produce (i.e. fruits and vegetables). A particular challenge for the produce industry is that *Listeria* spp. are commonly present on harvested crops, leading to a constant source of new contaminants. Therefore, the onus is on the industry to mitigate the establishment of these resident strains in produce handling and processing facilities (PHP). The objective of this study was to characterize *Listeria* spp. isolates (n = 113) previously isolated from five PHP facilities in the Pacific Northwest (PNW) using molecular serogrouping and antimicrobial resistance patterns. Most individual PHP facilities contained a single serogroup of *L. monocytogenes;* two facilities were positive for serogroup 1 only and two facilities were positive for serogroup 4 only. The facility with the highest prevalence of *Listeria* spp. was positive for both serogroups. All *Listeria* spp. isolates were sensitive to ampicillin, erythromycin, gentamicin, imipenem, co-trimoxazole, tetracycline and vancomycin and resistance to cefoxitin and nalidixic acid. High proportion (66%) of *Listeria* spp. isolates were resistant to clindamycin, whereas resistance to penicillin, ciprofloxacin, rifampicin, and novobiocin resistance was less commonly observed. Three *L.* monocytogenes isolates and one *L. innocua* isolate were determined to be multi-drug resistant. Strain characterization results demonstrated a high level of strain diversity in PHP facilities and persistent strains were not identified.

Keywords: Antimicrobial resistance, multi-drug resistance, serotyping, produce, *Listeria*

3.2 Introduction

Listeria spp. are widespread bacteria in the agricultural environment that have public health implications (Farber and Peterkin, 1991; Strawn et al., 2013). Multistate foodborne listeriosis outbreaks in the United States in the last ten years have been linked to produce contaminated with the pathogenic species *L. monocytogenes* (Angelo et al., 2017; Burall et al., 2017; McCollum et al., 2013). The most infamous example is the 2011 listeriosis outbreak linked to Jensen Farms whole cantaloupes. The outbreak stretched across 28 states, sickened 147, and was associated with 33 deaths (McCollum et al., 2013). The outbreak strain was isolated from numerous samples collected throughout the company's production environment (McCollum et al., 2013). Additional listeriosis outbreaks have identified the persistence of *L. monocytogenes* strains in food production and processing environments as the contributing factor for food contamination.

Basic characterization of *Listeria* spp. strains recovered from PHP facilities is useful to understand population and molecular diversity, relatedness, and pathogenicity, with keen emphasis on *L. monocytogenes* isolates. Of the many strategies available to characterize *Listeria* spp. two include serogrouping and antimicrobial resistance (AMR) profiling.

Together, these characterization methods are tools to facilitate isolate comparisons that evaluate the potential for persistent strains in PHP facilities. The primary objective of this study was to characterize *Listeria* spp. isolates previously isolated from five produce handling and processing (PHP) facility environments in the Pacific Northwest (PNW) (Jorgensen et al., 2019b) and to evaluate diversity and transient/persistent nature of *Listeria* isolates from these environments.

3.3 Materials and methods

3.3.1 Listeria *spp. isolate information*

Listeria spp. (n = 165) were previously isolated from environmental samples collected from produce handling and processing (PHP) facilities in the Pacific Northwest between May 2018 and April 2019 (Jorgensen et al., 2019b). Isolates were speciated using the Microgen® *Listeria*-ID system (Microgen; Microgen Bioproducts, Camberly, UK) and cryogenically preserved in tryptic soy broth (TSB, Neogen, Lansing, MI, USA) with 50% glycerol. Isolate metadata can be found in Appendix D.

3.3.2 Multiplex PCR serogrouping of Listeria monocytogenes *isolates*

Listeria monocytogenes isolates were revived from the freezer by direct transfer to tryptic soy agar + 0.6% yeast extract plates (TSAYE, Neogen, Lansing, MI, USA) with incubation at 35°C for 24 h. A single isolated colony was transferred to a microfuge tube containing 500 µl of Lucigen's QuickExtract™ DNA Extraction Solution (Lucigen Corporation, Middleton, WI, USA). Cell lysis was completed by subsequent incubations at 65°C for 6 min and 98°C for 2 min. The resulting lysates served as the DNA template (1 µl/reaction), and they were stored at -20°C for subsequent PCR reactions.

A modified version of the multiplex polymerase chain reaction (PCR) previously described by Doumith et al. 2004 (Doumith et al., 2004) was used to serogroup each isolate. The multiplex PCR mixture (25 µl) was formulated as 1 Unit of *Taq* DNA Polymerase (NEB, New England BioLabs, Ipswich, MA, USA), 1X PCR buffer mix (NEB), 200 µM dNTPs (NEB), 1 µM of each primer for *lmo0737*, ORF2819, and ORF2110, 1.5 µM of each primer for *lmo1118*, and 0.2 µM of each primer for *prs.* Primers were purchased from

Integrated DNA Technologies (IDT, Coralville, IA, USA) and DNA sequences are shown in Table 3.1.

Table 3.1. PCR target genes, primer sequences, and amplicon size for serogrouping *Listeria monocytogenes* (Adapted from Doumith et al, 2004).

Target	Primer Sequence (5'-3')	Product size (bp)	Serogroups resulting in PCR product
prs	prsF: 5'-GCTGAAGAGATTGCGAAAGAAG-3' prsR: 5'-CAAAGAAACCTTGGATTTGCGG-3'	370	*Listeria* spp.
ORF2819	ORF2819F: 5'-AGCAAAATGCCAAAACTCGT-3' ORF2819R: 5'-CATCACTAAAGCCTCCCATTG-3'	471	1/2b, 3b, 4b, 4d, 4e
ORF2110	ORF2110F: 5'-AGTGGACAATTGATTGGTGAA-3' ORF2110R: 5'-CATCCATCCCTTACTTTGGAC-3'	597	4b, 4d, 4e
lmo0737	lmo0737F: 5'-AGGGCTTCAAGGACTTACCC-3' lmo0737R: 5'-ACGATTTCTGCTTGCCATTC-3'	691	1/2a, 1/2c, 3a, 3c
lmo1118	lmo1118F: 5'-AGGGGTCTTAAATCCTGGAA-3' lmo1118R: 5'-CGGCTTGTTCGGCATACTTA-3'	906	1/2c, 3c

PCR was performed using the Applied Biosystems™ SimpliAmp™ thermocycler (Fisher Scientific, Waltham, MA, USA) with an initial denaturation step at 94°C for 3 min; 35 cycles of denaturation at 94°C for 24 s, annealing at 53°C for 75 s, and extension at 72°C for 75 s; and a final incubation at 72°C for 7 min. PCR amplification products (8 µl) were separated on a 2% UltraPure™ agarose gel containing Gel Red (Thermo Fisher Scientific, Waltham, MA, USA) in TBE buffer. Amplicon separation was achieved using a voltage gradient program of 45 min at 60 V, 80 V, and 100 V. PCR products were visualized using the Gel Doc™ XR+ Imager (Bio-Rad, Hercules, CA, USA). A 1-Kb DNA ladder (Thermo Fisher Scientific, Waltham, MA, USA) served as the size standard for each gel. Control strains for the following known *L. monocytogenes* serotypes were included in each gel: 1/2a, DE25-1; 1/2b, OE90-1; 1/2c, FF1-1 and OF64-2; and 4b, FF5-1.

3.3.3 Antimicrobial resistance by disk diffusion

AMR characteristics of *Listeria* spp. isolates were determined using methods previously described (Kovacevic et al., 2013b, Milillo, 2015). Briefly, each *Listeria* isolate was streaked for isolation on TSAYE (Neogen) and incubated at 35°C for 24 h. A single colony was transferred to TSB (3 ml; Neogen) and incubated at 35°C for 18 h with shaking (200 rpm). A 70 µl aliquot of the liquid culture was mixed with 7 mL of 0.75% agar tempered at 45°C and overlaid onto previously prepared Mueller-Hinton agar plates (MHA, Neogen). Antibiotic sensitivity disks (Table 3.2; Becton, Dickinson and Company (BD), Sparks, MD) were placed onto the surface of the solidified MHA plates and incubated at 35°C for 24 h. The diameter of each zone of inhibition was measured to the nearest mm. Interpretation of antibiotic susceptibility (sensitive, intermediate, resistant) was determined in accordance with Clinical Laboratory Standards Institute (CLSI, Wayne, PA, USA) criteria (Table 3.2). *Listeria* spp. isolates displaying resistance to specific antibiotics were verified by an additional 1-2 replications of the disk diffusion assay. *Escherichia coli* ATCC 35218 and *Staphylococcus aureus* ATCC 25923 were used as control cultures for the disk diffusion assay.

AMR patterns were compared for isolates recovered from the same swab sample. Isolates were considered to be unique strains if there was a difference of at least 3 mm for a single antibiotic or a difference of at least 2 mm for three or more antibiotics. Otherwise, isolates from the same sample were considered to be representative of a single strain (i.e., "clonal") and one was chosen as the representative strain for reporting purposes. When the inhibition zones of "identical" isolates spanned the resistance classification, the most resistant isolate was chosen. Of 165 *Listeria* spp. isolates screened, 52 were considered to

be “identical” to at least one other isolate from the same sample. Therefore, the remainder of this manuscript will focus on the 113 *Listeria* spp. strains that represent the diversity in isolates from PHP facilities.

Table 3.2. Panel of 18 antibiotics used to asses antimicrobial resistance of *Listeria* spp. recovered from Jorgensen et al, 2019, by disk diffusion. This table also includes antibiotic breakpoint ranges for control strains and break points for *Listeria* spp.

Antibiotic	Abbreviation	Antibiotic Disk Dose (µg)	*S. aureus*[1] ATCC 25923 range (mm)	*E. coli*[1] ATCC 25922 range (mm)	*Listeria* spp. breakpoints (mm)		
					Sensitive (S)	Intermediate (I)	Resistant (R)
Amikacin	AMK	30	20-26	19-26	≤ 14	15-16	≥ 17
Ampicillin	AMP	10	27-35	15-22	19	-[2]	20
Cefoxitin	FOX	30	23-29	23-29	14	15-17	18
Chloramphenicol	CHL	30	19-26	21-27	12	13-17	18
Ciprofloxacin	CIP	5	22-30	29-37	15	16-20	21
Clindamycin	CLI	2	24-30	N/A[3]	14	15-20	21
Erythromycin	ERY	15	22-30	N/A[3]	14	15-22	23
Gentamicin	GEN	10	19-27	19-26	12	13-14	15
Imipenem	IPM	10	N/A[3]	26-32	13	14-15	16
Kanamycin	KAN	30	19-26	17-25	13	14-17	18
Nalidixic acid	NAL	30	N/A[3]	22-28	13	14-18	19
Novobiocin	NOV	30	22-31	N/A[3]	17	18-21	22
Penicillin	PEN	10 U[4]	26-37	N/A[3]	19	20-27	28
Rifampin	RIF	5	26-34	8-10	16	17-19	20
Streptomycin	STR	10	14-22	12-20	11	12-14	15
Co-trimoxazole	SXT	1.25 / 23.75[5]	24-32	23-29	10	11-15	16
Tetracycline	TET	30	24-30	18-25	14	15-18	19
Vancomycin[6]	VAN	5	17-21	N/A[3]	9	-[2]	10

[1]Control strain ranges for each antibiotic determined from Clinical Laboratory Standards Institute (CLSI, Wayne, PA, USA).
[2]No intermediate breakpoint, only sensitive or resistant, (-).
[3]Breakpoints for *Listeria* spp. were determined from CLSI (Wayne, PA, USA). Nalidixic acid and streptomycin were used from those established for *Enterobacteriaceae*, vancomycin breakpoints were taken from those established from *Enterococcus*, while all others were used for *Staphylococcus*. Ranges or breakpoints not determined or available by CLSI guidelines, (N/A).
[4]Penicillin disk concentration in international units of penicillin (U).
[5]Co-trimoxazole is composed of two antibiotics, trimethoprim (1.25 µg) and sulfamethoxazole (23.75 µg).
[6]Vancomycin *Listeria* spp. breakpoints determined from Dalynn Biologicals (Calgary, AB, Canada).

3.4 Results and discussion

3.4.1 Serogroups of Listeria monocytogenes *isolated from PHP facilities*

Seventy-five unique *L. monocytogenes* isolates recovered from PHP facilities belonged to two molecular serogroups: serogroup 1 (1/2a, 3a) and serogroup 4 (4b, 4d, 4e). Twenty-three isolates (31%) belonged to serogroup 1 (lineage II) and 52 (69%) belonged to serogroup group 4 (lineage I) (Table 3.3).

Table 3.3. Prevalence of *Listeria monocytogenes* serogroups from produce handling and packing facilities (PHP) in the Pacific Northwest.

Facility	Serogroup	
	Group 1 (1/2a, 3a)	**Group 4 (4b, 4d, 4e)**
A	12/52 (23%)	40/52 (77%)
C	0/9 (0%)	9/9 (100%)
D	0/3 (0%)	3/3 (100%)
E	2/2 (100%)	0/3 (0%)
G	9/9 (100%)	0/9 (0%)
Total	23/75 (31%)	52/75 (69%)

Environmental sampling at most facilities (4 out of 5) resulted in the isolation of a single serogroup. For example, all *L. monocytogenes* isolates from Facilities E and G belonged to serogroup 1. Interestingly, *L. monocytogenes* was also the only *Listeria* spp. recovered from environmental samples in these two facilities. Facilities E and G are very similar in the types of commodities that they process (mostly berries) and their general processing technique. Two other PHP facilities (C and D) only had *L. monocytogenes* isolates that belonged to serogroup 4. Environmental sampling of these two facilities also resulted in the recovery of *L. innocua* isolates. These two facilities regularly work together,

work with the same commodities, and transport product back and forth between the two facilities. One PHP facility, Facility A, was a packinghouse that handled and packed a large variety of fresh produce. The prevalence of *Listeria* spp. in this facility's environmental sampling was quite high (>26%), including numerous *Listeria* species (*L. monocytogenes, L. innocua, L. ivanovii,* and *L. welshimeri*). Therefore, it is not surprising that more than one serogroup of *L. monocytogenes* (1 and 4) was present in the facility. Serogroup 4 was more frequently isolated (40/52) than serogroup 1 (12/52) at Facility A. No *L. monocytogenes* isolates from serogroups 2 (1/2c, 3c) and 3 (1/2b, 3b, 7) were recovered from PHP facilities.

Previous studies have also reported *L. monocytogenes* serotypes from lineage I (1/2b, 3b, 3c, 4b) and II (1/2a, 1/2c, 3a) as the most common lineages recovered from foods and food production environments (Orsi et al., 2011). Gianfranceshci et al. (2003) found *L. monocytogenes* serotypes 4b, 1/2a, 1/2b, and 1/2c to be the most commonly isolated serotypes from a variety of foods and food production facilities in Italy. These serotypes were also dominant in food products from China (Chen et al., 2009) and in vegetable salads in Chile and the United Kingdom (Cordano and Jacquet, 2009; Little et al., 2007). Sauders et al. (2009) reported the persistence of *L. monocytogenes* in retail establishments which included environmental samples from several produce-associated areas. This study reported 29 isolates were from lineage I (29/34; 85%) and 5 isolates were from lineage II (5/34; 14.7%) (Sauders et al., 2009). A three year study of the prevalence of *L. monocytogenes* in 54 small food businesses in Ireland, including facilities that worked with produce, recovered and separated 255 isolates using the Doumith et al. (2004) method (Leong et al., 2017). In total 112 isolates from food and environmental

samples were separated into serogroup 1 (43.9%), 70 were in serogroup 4 (27.5%), 41 were in serogroup 2 (16.1%), and 31 were in serogroup 3 (12.2%) (Leong et al., 2017).

The current study did not identify any *L. monocytogenes* isolates from serogroups 2 (1/2c, 3c) and 3 (1/2b, 3b, 7). Fox et al. (2012) and Kovacevic et al. (2013a) recovered and serotyped 222 and 54 *L. monocytogenes* isolates, respectively. Collectively, serotype 1/2c was identified in 31/276 (11.2%) isolates, 23/276 (8.3%) were 1/2b, and 1/276 (<1%) were 3b. No isolates of serotypes 3c or 7 were recovered in either study (Fox et al., 2012; Kovacevic et al., 2013a).

L. monocytogenes serotypes 1/2a (serogroup 1) and serotype 4b (serogroup 4) are responsible for the majority foodborne listeriosis outbreaks (Farber and Peterkin, 1991; Vazquez-Boland et al., 2001), including those associated with produce (Garner and Kathariou, 2016; Zhu et al., 2017). Notably, the largest listeriosis outbreak in U.S. was linked to whole cantaloupes contaminated with *L. monocytogenes* serotypes 1/2a and 1/2b (McCollum et al., 2013), and *L. monocytogenes* serotype 4b was linked to a multistate outbreak associated with caramel apples (Angelo et al., 2017). Serotype 4b strains similar in virulence to the caramel apple outbreak strain were also recovered from nectarines, peaches, bagged lettuce, cheeses, meats and more human foodborne listeriosis patients in U.S. through 2015 (Burall et al., 2017). The initial serogroup classification (serogroups 1 and 4) of the PHP isolates suggests that further analysis of these isolates is necessary to confirm serotype level classification and potential pathogenicity.

3.4.2 Sensitivity and resistance in all Listeria *spp. to a select group of antibiotics*

All *Listeria* spp. isolates (n = 113) from PHP facilities were sensitive to AMP, ERY, GEN, IMP, SXT, TET, and VAN and resistant to FOX and NAL (data not shown). Previous studies have demonstrated consistent sensitivity to the same seven antibiotics (Kovacevic et al., 2013b). AMP and GEN, and other beta-lactams, commonly show sensitivity or intermediate sensitivity due to the high affinity of penicillin binding protein 3 (PBP3) in *Listeria* spp. membranes (Hof et al., 1997). ERY, TET, IMP, SXT, VAN are bacteriostatic agents that are commonly affective against *Listeria* spp. (Hof et al., 1997).

Studies have described natural resistance in *Listeria* spp. to FOX and NAL from several sources (food, food production environments, clinical listeriosis cases). FOX resistance is due to the low affinity for PBP3 (Hof et al., 1997). NAL, a part of the quinolones antimicrobial class, has limited activity against Gram-positive bacteria, such as *Listeria* spp. (Hof et al., 1997).

3.4.3 Antimicrobial resistance to CIP, CLI, NOV, PEN, and RIF and strain diversity

AMR of *Listeria* spp. varied across facilities. All *Listeria* spp. isolates were resistant or of intermediate sensitivity to CLI and PEN (Figure 1). CLI resistance was the most common, at 66% (75/113), and present in at least two isolates in all PHP facilities. PEN resistance was the next most common, seen in 33 (29%) isolates from three PHP facilities (A, C, and G). PEN resistance was nearly always associated with CLI resistance; with 27/33 CLI resistant isolates also possessing resistance to PEN.

Notably, *Listeria* spp. strains recovered from all PHP facilities covered all resistance classes (resistant, intermediate, sensitive) for CIP, RIF, and NOV; however, resistance to

these antibiotics was uncommon in the isolate set. All CIP resistant isolates (11/113, 10%) were *L. monocytogenes* strains recovered from Facilities C and G only. Isolates from *L. monocytogenes* serogroup 1 (39%) were more likely to carry CIP resistance than isolates from serogroup 4 (4%). The four RIF resistant isolates (3.5%) were all *L. innocua*, recovered from Facility E; these isolates were also resistant to CLI. Five isolates (4.4%) demonstrated resistance to NOV and were recovered from Facilities A and G. NOV resistance was observed in three *L. monocytoge*nes serogroup 4 isolates from Facility A, whereas Facility G had two *L. innocua* isolates resistant to NOV. Four of these isolates were resistant to CLI, PEN, and NOV, indicating a low level of strains meeting the classification of multi-drug resistant (MDR).

NOV resistance and potential resistance mechanisms has been reported rarely in *Listeria* spp. food chain isolates. Wong et al. (1990) found only 1/356 (0.28%) of *L. monocytogenes* isolates from different food types in Taiwan resistant to NOV, while Purwati et al. (2003) reported that 57.1% (16/28) of *L. monocytogenes* strains isolated from chicken meat in Malaysia possessing NOV resistance. While more data are needed on NOV resistance in *Listeria* spp. food chain isolates, NOV is not a common treatment option for human listeriosis (Temple and Nahata, 2000), therefore it is rarely included *Listeria* spp. AMR screening panels.

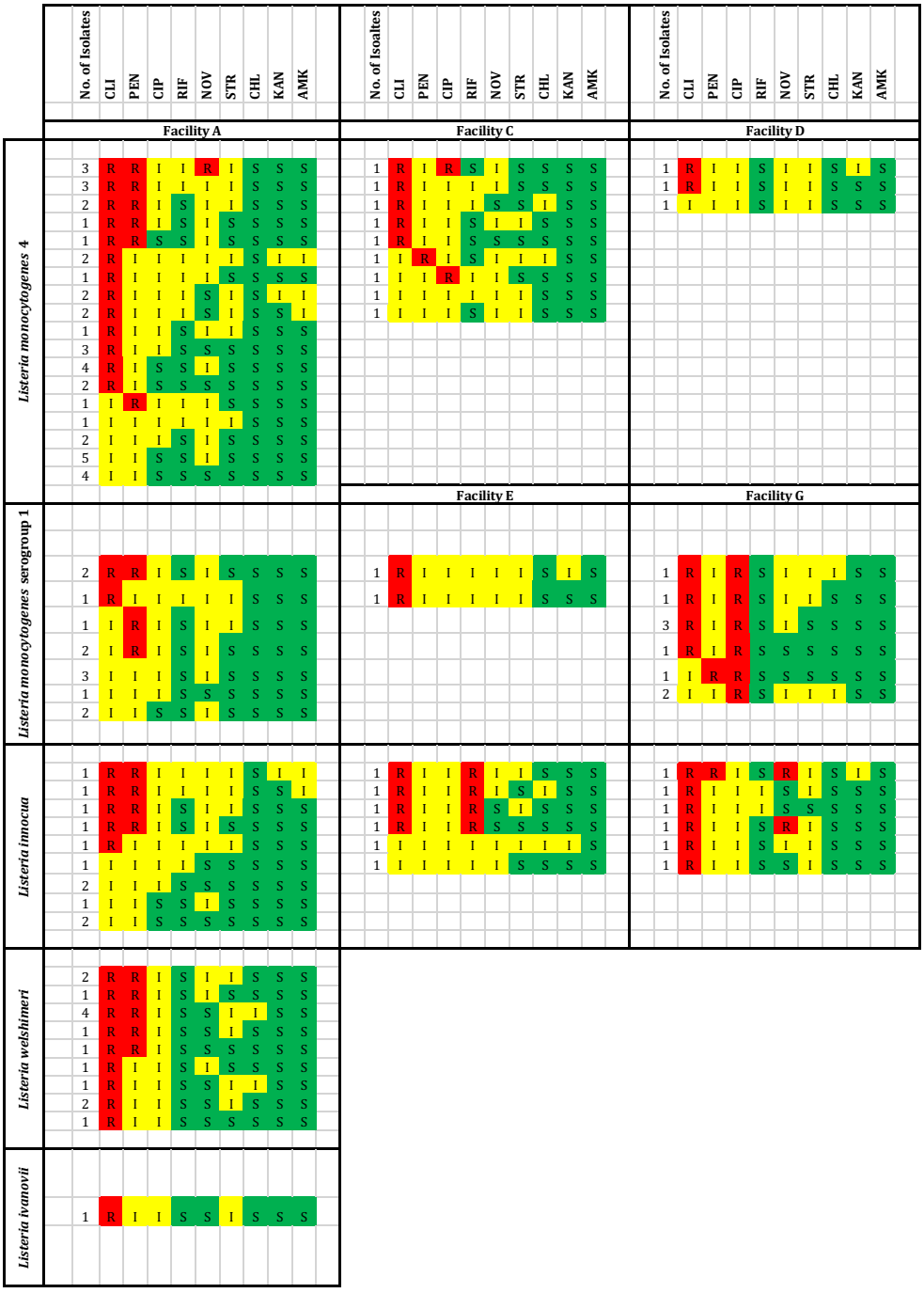

Figure 3.1. Antimicrobial resistance patterns of recovered *Listeria* spp. isolates. 113 isolates from five (A, C, D, E and G) Pacific Northwest PHP facilities were separated into strains with matching AMR patterns based on nine antibiotics. The number (No.) of isolates is in the white column with determined and matching AMR profiles in the next nine columns. Recovered species is in the far-left column. Strains are separated by facility and into *L. monocytogenes* serogroups (1 or 4) or other *Listeria* species. Red "R" indicates resistance, yellow "I" indicates intermediate sensitivity and green "S" indicates sensitivity to that antibiotic.

No *L. monocytogenes* isolates in the current study were resistant to RIF (Figure 3.1). RIF is one of the current treatment options for listeriosis infections in humans (Olaimat et al., 2018). Conter et al. (2009) observed RIF resistance in 1.6% of *L. monocytogenes* recovered from food and food production environments, all of which came from meat products. Literature on the mechanisms of RIF resistance for *L. monocytogenes* from food chain isolates is scarce. In 2014, a clinical case was reported with *L. monocytogenes* strain isolated from a listeriosis patient possessing high resistance to RIF due to mutations in the *rpoB gene*, which encodes a β-subunit of the RNA polymerase (Chenal-Francisque et al., 2014). Troxler et al. (2000) originally reported that all *Listeria* spp. are naturally sensitive to RIF; however, we found four *L. innocua* isolates with RIF resistant profiles. Because *L. monocytogenes* and *L. innocua* are closely related species (Orsi et al., 2011), transfer of genes conferring resistance may be possible, though it has not been previously associated with RIF resistance. Moreover, observing RIF resistance in *L. innocua* and not in any other species points to species specific resistance (Walsh et al., 2001). These isolates all came from a facility that washes and packs a variety of produce year-round, with minimal regulatory requirements on the production environment cleaning and sanitation. The production environment could be a factor influencing the resistance profiles we have observed throughout this study, though there is presently no evidence in the literature to support this.

3.4.4 MDR and emerging resistance

Listeria spp. isolates from PHP facilities were mostly sensitive to AMK (105/113; 93%), KAN (105/113; 93%), CHL (101/113; 89%), and STR (59/113; 52%). These trends are important in being able to notice potential emerging resistance. Nearly all *Listeria* spp. isolates were sensitive to AMK; however, there were eight isolates with intermediate resistance; all from a single PHP facility (Facility A). Of particular concern is that isolates that were intermediately sensitive to AMK were often only sensitive to one or two of the antibiotics tested. For example, three isolates from Facility A were sensitive only to CHL and were intermediately resistant or resistant to all other antibiotics tested. Although rare, AMK resistance has been reported in few studies (Arslan, 2007; Rota et al., 1996), and should be continuously monitored in *Listeria* spp. food chain isolates.

Facility A had the only *L. monocytogenes* isolates with MDR resistance (resistant to three or more antibiotics) (Figure 3.1). These three isolates were all serogroup 4 strains and demonstrated resistance to CLI, NOV and PEN. We believe this to be the first report of *L. monocytogenes* with this combination of MDR. All three isolates were recovered from environmental samples collected on the same day. These isolates were recovered inside the Facility A, specifically from swab samples of the floor inside an entry point, another floor location near that door, and a trench drain. These isolates were collected on a high production volume day of cabbage-handling; however, the spread of a MDR strain throughout a large surface area of the production facility is concerning. A similar study by Prazak et al. (2001) in Texas cabbage packing sheds found 17/21 isolates had resistance to three or more antibiotics, with one isolate being resistant to eight different antibiotics (CLI, cephalothin, CIP, ERY, tobramycin, oxacillin, PEN, STR). Almost all *L. monocytogenes* MDR

strains (16/18, 88.9%) in the Texas study showed resistance to CLI and PEN, which is similar to our results, where 27/39 (69.2%) MDR isolates also possessed CLI and PEN resistance. The study by Prazak et al. (2001) did not include NOV in its antibiotic panel. Furthermore, it is unclear if there is a MDR relationship between these three antibiotics (CLI, PEN, NOV) based on current literature.

Resistance to other antimicrobials, including sanitizers in food processing industry, has received increased attention, with concerns that strains possessing AMR may also be more likely to form biofilms and persist in food facilities (Carpentier and Chassaing, 2004; Colagiorgi et al., 2017). However, to our knowledge, definite links between antibiotic resistance of *Listeria* spp. and increased tolerance or resistance to any particular sanitizer class have not been reported. Genetic sequencing data are needed to investigate mechanisms behind AMR resistance observed here, in addition to targeted studies addressing differences in species or serogroup resistance, and potential for co-selection or co-resistance among antibiotics screened here and other antimicrobials.

3.5 Conclusions

A highly diverse population of *Listeria* spp. can be found during active operations in PHP facilities. This study demonstrated diversity on species and serotype levels. In addition, the AMR profiles suggested a high degree of genetic variability within facilities and across facilities. These data confirmed that the risk of *Listeria* spp., and specifically *L. monocytogenes*, to enter PHP facilities, particularly packinghouses, is high; however, strain persistence in these facilities was not clearly demonstrated. A rigorous cleaning and sanitation schedule for food contact surfaces and control of traffic patterns of mobile

elements is necessary to minimize the potential for cross-contamination and the establishment of persistent *Listeria* spp. The overall strain diversity and observation that most isolates with matching AMR patterns came from the same sampling day suggest that strains are transient, though further genetic confirmation is required to support this hypothesis. Overall, AMR was rare except for resistance to CLI and PEN. Our data, in conjunction with results from several other studies showing a high percentage of *Listeria* spp. isolates resistant to CLI, suggest a potential emerging resistance to CLI in *Listeria* spp. Resistance to PEN and RIF were surprising, as both of these antibiotics have been known to work effectively against listeriosis infection in humans. MDR resistance could be an indication that biofilms are present containing *Listeria* spp. with higher degree of antimicrobial tolerance. Although only observed on three occasions in Facility A, isolates with matching AMR patterns were recovered from different days. This may indicate the presence of an established strain, though further genomic characterization is needed, supplemented with collection of samples over a longer, consistent, period.

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3.7 References

Angelo, K. M., Conrad, A.R., Saupe, A., Dragoo, H., West, N., Sorenson, A., Barnes, A., Doyle, M., Beal, J., Jackson, K.A., Stroika, S., Tarr, C., Kucerova, Z., Lance, S., Gould, L.H., Wise, M., Jackson, B.R., 2017. Multistate outbreak of *Listeria monocytogenes* infections linked to whole apples used in commercially produced, prepackaged caramel apples: United States, 2014-2015. Epidemiol. Infect. 145. https://doi.org/10.1017/S0950268816003083

Arslan, S., Fatma, Ö., 2008. Prevalence and antimicrobial resistance of *Listeria* spp. in homemade white cheese. Food Control 19, 360–363. https://doi.org/10.1016/j.foodcont.2007.04.009

Burall, L.S., Grim, C.J., Datta, A.R., 2017. A clade of *Listeria monocytogenes* serotype 4b variant strains linked to recent listeriosis outbreaks associated with produce from a defined geographic region in the US. PLoS One 12, e0176912. https://doi.org/10.1371/journal.pone.0176912

Carpentier, B., Chassaing, D., 2004. Interactions in biofilms between *Listeria monocytogenes* and resident microorganisms from food industry premises. Int. J. Food Microbiol. 97, 111–122. https://doi.org/10.1016/J.IJFOODMICRO.2004.03.031

Chen, J., Zhang, X., Mei, L., Jiang, L., Fang, W., 2009. Prevalence of *Listeria* in chinese food products from 13 provinces between 2000 and 2007 and virulence characterization of *Listeria monocytogenes* isolates. Foodborne Pathog. Dis. 6, 7–14. https://doi.org/10.1089/fpd.2008.0139

Chenal-Francisque, V., Charlier, C., Mehvish, S., Dieye, H., Leclercq, A., Courvalin, P., Lecuit, M., 2014. Highly rifampin-resistant *Listeria monocytogenes* isolated from a patient with prosthetic bone infection. Antimicrob. Agents Chemother. 58, 1829–1830. https://doi.org/10.1128/AAC.02449-13

Colagiorgi, A., Bruini, I., Di Ciccio, P.A., Zanardi, E., Ghidini, S., Ianieri, A., Colagiorgi, A., Bruini, I., Di Ciccio, P.A., Zanardi, E., Ghidini, S., Ianieri, A., 2017. *Listeria monocytogenes* biofilms in the wonderland of food industry. Pathogens 6, 1–9. https://doi.org/10.3390/pathogens6030041

Cordano, A.M., Jacquet, C., 2009. *Listeria monocytogenes* isolated from vegetable salads sold at supermarkets in Santiago, Chile: prevalence and strain characterization. Int. J. Food Microbiol. 132, 176–179. https://doi.org/10.1016/j.ijfoodmicro.2009.04.008

Doumith, M., Buchrieser, C., Glaser, P., Jacquet, C., Martin, P., 2004. Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. J. Clin. Microbiol. 42, 3819–3822. https://doi.org/10.1128/JCM.42.8.3819-3822.2004

Farber, J.M., Peterkin, P.I., 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. 55, 476–511.

Fox, E.M., Delappe, N., Garvey, P., Mckeown, P., Cormican, M., Leonard, N., Jordan, K., 2012. PFGE analysis of *Listeria monocytogenes* isolates of clinical, animal, food and environmental origin from Ireland. J. Med. Microbiol. 61, 540–547. https://doi.org/10.1099/jmm.0.036764-0

Garner, D., Kathariou, S., 2016. Fresh produce–associated listeriosis outbreaks, sources of concern, teachable moments, and insights. J. Food Prot. 79, 337–344. https://doi.org/10.4315/0362-028X.JFP-15-387

Hof, H., Nichterlein, T., Kretschmar, M., 1997. Management of listeriosis. Clin. Microbiol. Rev. 10, 345–357.

Jorgensen, J., Waite-Cusic, J., Kovacevic, J., 2019b. Prevalence of *Listeria* spp. in Produce Handling and Processing Facilities in the Pacific Northwest.

Kovacevic, J., Arguedas-Villa, C., Wozniak, A., Tasara, T., Allen, K.J., 2013a. Examination of food chain-derived *Listeria monocytogenes* strains of different serotypes reveals considerable diversity in inlA genotypes, mutability, and adaptation to cold temperatures. Appl. Environ. Microbiol. 79, 1915–1922. https://doi.org/10.1128/AEM.03341-12

Kovacevic, J., Sagert, J., Wozniak, A., Gilmour, M.W., Allen, K.J., 2013b. Antimicrobial resistance and co-selection phenomenon in *Listeria* spp. recovered from food and food production environments. Food Microbiol. 34, 319–327. https://doi.org/10.1016/J.FM.2013.01.002

Leong, D., Nicaogáin, K., Luque-Sastre, L., Mcmanamon, O., Hunt, K., Alvarez-Ordóñez, A., Scollard, J., Schmalenberger, A., Fanning, S., O'byrne, C., Jordan, K., 2017. A 3-year multi-food study of the presence and persistence of *Listeria monocytogenes* in 54 small food businesses in Ireland. Int. J. Food Microbiol. 249, 18–26. https://doi.org/10.1016/j.ijfoodmicro.2017.02.015

Little, C., Taylor, F., Sagoo, S., Gillespie, I., Grant, K., McLauchlin, J., 2007. Prevalence and level of *Listeria monocytogenes* and other *Listeria* species in retail pre-packaged mixed vegetable salads in the UK. Food Microbiol. 24, 711–717. https://doi.org/10.1016/j.fm.2007.03.009

Liu, D., 2006. Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. J. Med. Microbiol. 55, 645–659. https://doi.org/10.1099/jmm.0.46495-0

McCollum, J.T., Cronquist, A.B., Silk, B.J., Jackson, K.A., O'Connor, K.A., Cosgrove, S., Gossack, J.P., Parachini, S.S., Jain, N.S., Ettestad, P., Ibraheem, M., Cantu, V., Joshi, M., DuVernoy, T., Fogg, N.W., Gorny, J.R., Mogen, K.M., Spires, C., Teitell, P., Joseph, L.A., Tarr, C.L., Imanishi, M., Neil, K.P., Tauxe, R. V., Mahon, B.E., 2013. Multistate outbreak of listeriosis associated with cantaloupe. N. Engl. J. Med. 369, 944–953. https://doi.org/10.1056/NEJMoa1215837

Milillo, M.S., 2015. Resistance to food processing sanitizers and heavy metals in *Listeria monocytogenes* from British Columbia, Canada and antibiogram profiles of clinically relevant Listeria monocytogenes from British Columbia and Alberta, Canada. University of British Columbia. https://doi.org/10.14288/1.0165806

Olaimat, A.N., Al-Holy, M.A., Shahbaz, H.M., Al-Nabulsi, A.A., Abu Ghoush, M.H., Osaili, T.M., Ayyash, M.M., Holley, R.A., 2018. Emergence of antibiotic resistance in *Listeria monocytogenes* isolated from food products: a comprehensive review. Compr. Rev. Food Sci. Food Saf. 17, 1277–1292. https://doi.org/10.1111/1541-4337.12387

Orsi, R.H., Bakker, H.C. de., Wiedmann, M., 2011. *Listeria monocytogenes* lineages: genomics, evolution, ecology, and phenotypic characteristics. Int. J. Med. Microbiol. 301, 79–96. https://doi.org/10.1016/j.ijmm.2010.05.002

Rota, C., Yanguela, J., Blanco, D., Carraminana, J.J., Arino, A., Herrera, A., 1996. High prevalence of multiple resistance to antibiotics in 144 *Listeria* isolates from Spanish dairy and meat products. J. Food Prot. 59, 938–943. https://doi.org/10.4315/0362-028X-59.9.938

Sauders, B.D., Sanchez, M.D., Rice, D.H., Corby, J., Stich, S., Fortes, E.D., Roof, S.E., Wiedmann, M., 2009. Prevalence and molecular diversity of *Listeria monocytogenes* in retail establishments. J. Food Prot. 72, 2337–2349. https://doi.org/10.4315/0362-028X-72.11.2337

Strawn, L.K., Gröhn, Y.T., Warchocki, S., Worobo, R.W., Bihn, E.A., Wiedmann, M., 2013. Risk factors associated with *Salmonella* and *Listeria monocytogenes* contamination of produce fields. Appl. Environ. Microbiol. 79, 7618–7627. https://doi.org/10.1128/AEM.02831-13

Temple, M.E., Nahata, M.C., 2000. Treatment of listeriosis. Ann. Pharmacother. 34, 656–661. https://doi.org/10.1345/aph.19315

Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J., Kreft, J., 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. 14, 584–640. https://doi.org/10.1128/CMR.14.3.584-640.2001

Walsh, D., Duffy, G., Sheridan, J.J., Blair, I.S., McDowell, D.A., 2001. Antibiotic resistance among *Listeria*, including *Listeria monocytogenes*, in retail foods. J. Appl. Microbiol. 90, 517–522. https://doi.org/10.1046/j.1365-2672.2001.01273.x

Zhu, Q., Gooneratne, R., Hussain, M.A., 2017. *Listeria monocytogenes* in fresh produce: outbreaks, prevalence and contamination levels. Foods 6, 1–11. https://doi.org/10.3390/foods6030021

Overall conclusions

The knowledge and data gained from this study provides the produce industry with valuable information on the prevalence of *Listeria* spp. in the Pacific Northwest. This study reports that *Listeria* spp. prevalence is facility specific and that *L. monocytogenes* was found in all facilities positive for *Listeria* spp. Facilities that handle raw product may have increased prevalence of *Listeria* spp. due to the embedded nature of production, requiring high traffic in and out of these facility types. It is unclear if there is a potential for cross-contamination in these facilities but it important for these facilities to acknowledge and mitigate these areas that are potentially conducive to *Listeria* spp. hotspots. Being able to isolate and track strains throughout a food production facility can be a very useful tool in understanding contamination patterns and potential strain persistence.

Strain characterization data from our study highlight that *L. monocytogenes* recovered may be subtypes that are of public health concern. It is reassuring that all *Listeria* spp. strains recovered showed susceptibility to clinically relevant antibiotics. It is worth noting, though, that there was a relatively high amount of antimicrobial resistance diversity associated with several other antibiotics, emerging resistance, and isolates that showed resistance in up to three antibiotics. Further characterization of *L. monocytogenes* is necessary to be able to understand the pathogenic potential of strains recovered from this study. In conclusion, environmental monitoring and strain characterization in produce handling and processing environments in the Pacific Northwest is absolutely necessary to be able to control and further understand foodborne pathogens like *L. monocytogenes*.

Bibliography

Angelo, K. M., Conrad, A.R., Saupe, A., Dragoo, H., West, N., Sorenson, A., Barnes, A., Doyle, M., Beal, J., Jackson, K.A., Stroika, S., Tarr, C., Kucerova, Z., Lance, S., Gould, L.H., Wise, M., Jackson, B.R., 2017. Multistate outbreak of *Listeria monocytogenes* infections linked to whole apples used in commercially produced, prepackaged caramel apples: United States, 2014-2015. Epidemiol. Infect. 145. https://doi.org/10.1017/S0950268816003083

Arnade, C., Calvin, L., Kuchler, F., 2009. Consumer response to a food safety shock: the 2006 food-borne illness outbreak of *E. coli* O157: H7 linked to spinach. Rev. Agric. Econ. 31, 734–750. https://doi.org/10.1111/j.1467-9353.2009.01464.x

Arslan, S., Fatma, Ö., 2008. Prevalence and antimicrobial resistance of *Listeria* spp. in homemade white cheese. Food Control 19, 360–363. https://doi.org/10.1016/j.foodcont.2007.04.009

Barton Behravesh, C., Jones, T.F., Vugia, D.J., Long, C., Marcus, R., Smith, K., Thomas, S., Zansky, S., Fullerton, K.E., Henao, O.L., Scallan, E., 2011. Deaths associated with bacterial pathogens transmitted commonly through food: foodborne diseases active surveillance network (FoodNet), 1996–2005. J. Infect. Dis. 204, 263–267. https://doi.org/10.1093/infdis/jir263

Berrang, M.E., Frank, J.F., 2012. Generation of airborne *Listeria innocua* from model floor drains. Food Prot. 75, 1328–1331. https://doi.org/10.4315/0362-028X.JFP-12-021

Beuchat, L.R., 1996. Pathogenic microorganisms associated with fresh produce. J. Food Prot. 59, 204–216. https://doi.org/10.4315/0362-028X-59.2.204

Bierschwale, S., Blackman, S., Butts, J.N., Carter, D., Coles, C., Crawford, W.M., Denault-Bryce, P., Eisenberg, B.A., Estrada, M., Ewell, H., Foster, S., Hardin, M., Hau, H., Kerr, J., Mills, B., Owens, E.M., Parker, C., Petran, R.L., Prince, G., Raede, J., Roberson, M., Shergill, G., Snyder, K., Stoltenberg, S.K., Suslow, T., Special, B.Z., Butts, J., 2013. Guidance on Environmental Monitoring and Control of Listeria for the Fresh Produce Industry. United Fresh.

Borucki, M.K., Call, D.R., 2003. *Listeria monocytogenes* serotype identification by PCR. J. Clin. Microbiol. 41, 5537–5540. https://doi.org/10.1128/JCM.41.12.5537-5540.2003

Buchrieser, C., Brosch, R., Catimel, B., Rocourt, J., 1993. Pulsed-field gel electrophoresis applied for comparing *Listeria monocytogenes* strains involved in outbreaks. Can. J. Microbiol. 39, 395–401. https://doi.org/10.1139/m93-058

Burall, L.S., Grim, C.J., Datta, A.R., 2017. A clade of *Listeria monocytogenes* serotype 4b variant strains linked to recent listeriosis outbreaks associated with produce from a defined geographic region in the US. PLoS One 12, e0176912. https://doi.org/10.1371/journal.pone.0176912

Carpentier, B., Cerf, O., 2011. Review — Persistence of *Listeria monocytogenes* in food industry equipment and premises. Int. J. Food Microbiol. 145, 1–8. https://doi.org/10.1016/j.ijfoodmicro.2011.01.005

Carpentier, B., Chassaing, D., 2004. Interactions in biofilms between *Listeria monocytogenes* and resident microorganisms from food industry premises. Int. J. Food Microbiol. 97, 111–122. https://doi.org/10.1016/J.IJFOODMICRO.2004.03.031

CDC, 2017a. FastStats - Life Expectancy. https://www.cdc.gov/nchs/fastats/life-expectancy.htm (accessed 8.30.19).

CDC, 2017b. Mann Packing Recalls Minimally Processed Vegetable Products Because of Possible Health Risk | FDA. https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts/mann-packing-recalls-minimally-processed-vegetable-products-because-possible-health-risk (accessed 8.30.19).

CDC, 2016a. Multistate outbreak of listeriosis linked to frozen vegetables. https://www.cdc.gov/listeria/outbreaks/frozen-vegetables-05-16/index.html (accessed 29 August 2019).

CDC, 2016b. Multistate outbreak of listeriosis linked to packaged salads produced at Springfield, Ohio Dole processing facility. https://www.cdc.gov/listeria/outbreaks/bagged-salads-01-16/index.html (accessed 29 August 2019).

CDC, 2015. Multistate outbreak of listeriosis linked to commercially produced, prepackaged caramel apples made from Bidart Bros. apples. https://www.cdc.gov/listeria/outbreaks/caramel-apples-12-14/index.html (accessed 29 August 2019).

CDC, 2012. Multistate outbreak of listeriosis linked to whole cantaloupes from Jensen Farms, Colorado. https://www.cdc.gov/listeria/outbreaks/cantaloupes-jensen-farms/index.html (accessed 29 August 2019).

CDC, 2006. *E. coli* outbreak from fresh spinach. https://www.cdc.gov/ecoli/2006/spinach-10-2006.html (accessed 29 August 2019).

Chan, Y.C., Wiedmann, M., 2008. Physiology and genetics of *Listeria monocytogenes* survival and growth at cold temperatures. Crit. Rev. Food Sci. Nutr. 49, 237–253. https://doi.org/10.1080/10408390701856272

Chapin, T.K., Nightingale, K.K., Worobo, R.W., Wiedmann, M., Strawn, L.K., 2014. Geographical and meteorological factors associated with isolation of *Listeria* species in New York state produce production and natural environments. J. Food Prot. 77, 1919–1928. https://doi.org/10.4315/0362-028X.JFP-14-132

Chen, J., Zhang, X., Mei, L., Jiang, L., Fang, W., 2009. Prevalence of *Listeria* in chinese food products from 13 provinces between 2000 and 2007 and virulence characterization of *Listeria monocytogenes* isolates. Foodborne Pathog. Dis. 6, 7–14. https://doi.org/10.1089/fpd.2008.0139

Chenal-Francisque, V., Charlier, C., Mehvish, S., Dieye, H., Leclercq, A., Courvalin, P., Lecuit, M., 2014. Highly rifampin-resistant *Listeria monocytogenes* isolated from a patient with prosthetic bone infection. Antimicrob. Agents Chemother. 58, 1829–1830. https://doi.org/10.1128/AAC.02449-13

Clark, C.G., Farber, J., Pagotto, F., Ciampa, N., Dore, K., Nadon, C., Bernard, K., Ng, L.-K., 2010. Surveillance for *Listeria monocytogenes* and listeriosis, 1995–2004. Epidemiol. Infect. 138, 559–572. https://doi.org/10.1017/S0950268809990914

Colagiorgi, A., Bruini, I., Di Ciccio, P.A., Zanardi, E., Ghidini, S., Ianieri, A., Colagiorgi, A., Bruini, I., Di Ciccio, P.A., Zanardi, E., Ghidini, S., Ianieri, A., 2017. *Listeria monocytogenes* biofilms in the wonderland of food industry. Pathogens 6, 1–9. https://doi.org/10.3390/pathogens6030041

Cordano, A.M., Jacquet, C., 2009. *Listeria monocytogenes* isolated from vegetable salads sold at supermarkets in Santiago, Chile: prevalence and strain characterization. Int. J. Food Microbiol. 132, 176–179. https://doi.org/10.1016/j.ijfoodmicro.2009.04.008

Desai, A.N., Anyoha, A., Madoff, L.C., Lassmann, B., 2019. Changing epidemiology of *Listeria monocytogenes* outbreaks, sporadic cases, and recalls globally: a review of ProMED reports from 1996 to 2018. Int. J. Infect. Dis. 84, 48–53. https://doi.org/10.1016/J.IJID.2019.04.021

Doumith, M., Buchrieser, C., Glaser, P., Jacquet, C., Martin, P., 2004. Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. J. Clin. Microbiol. 42, 3819–3822. https://doi.org/10.1128/JCM.42.8.3819-3822.2004

Dowe, M.J., Jackson, E.D., Morl, J.G., Bell', C.R., 1997. *Listeria monocytogenes* survival in soil and incidence in agricultural soils. J. Food Prot. 60, 1201–1207.

El-Shenawy, M.A., 1998. Sources of *Listeria* spp. in domestic food processing environments. Int. J. Environ. Health Res. 8, 241–251. https://doi.org/10.1080/09603129873516

Embarek, B., 1994. Presence, detection and growth of *Listeria monocytogenes* in seafoods: a review. Int. J. Food Microbiol. 23, 17–34. https://doi.org/10.1016/0168-1605(94)90219-4

Enright, M.C., Spratt, B.G., 1999. Multilocus sequence typing. Trends Microbiol. 7, 482–487. https://doi.org/10.1016/S0966-842X(99)01609-1

Farber, J.M., Peterkin, P.I., 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. 55, 476–511.

Farber, J.M., Peterkin, P.I., 1999. Incidence and behavior of *Listeria monocytogenes* in meat products. In: Ryser, E.T., Marth, E.H. (Eds.), *Listeria*, Listeriosis and Food Safety, 2nd Edition. Marcel Dekker, New York, pp. 505–564.

FDA, 2019. Recalls, market withdrawals, & safety alerts. https://www.fda.gov/safety/recalls-market-withdrawals-safety-alerts (accessed 30 August 2019).

FDA, 2017. Draft guidance for industry: Control of *Listeria monocytogenes* in ready-to-eat foods. https://www.fda.gov/regulatory-information/search-fda-guidance-documents/draft-guidance-industry-control-listeria-monocytogenes-ready-eat-foods (accessed 30 August 2019).

Fenlon, D.R., Wilson, J., Donachie, W., 1996. The incidence and level of *Listeria monocytogenes* contamination of food sources at primary production and initial processing. J. Appl. Bacteriol. 81, 641–650

Ferreira, V., Wiedmann, M., Teixeira, P., Stasiewicz, M.J., 2014. *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. J. Food Prot. 77, 150–170. https://doi.org/10.4315/0362-028X.JFP-13-150

Fox, E.M., Delappe, N., Garvey, P., Mckeown, P., Cormican, M., Leonard, N., Jordan, K., 2012. PFGE analysis of *Listeria monocytogenes* isolates of clinical, animal, food and environmental origin from Ireland. J. Med. Microbiol. 61, 540–547. https://doi.org/10.1099/jmm.0.036764-0

Freitag, N.E., Port, G.C., Miner, M.D., 2009. *Listeria monocytogenes* - from saprophyte to intracellular pathogen. Nat. Rev. Microbiol. 7, 623–628. https://doi.org/10.1038/nrmicro2171

Garner, D., Kathariou, S., 2016. Fresh produce–associated listeriosis outbreaks, sources of concern, teachable moments, and insights. J. Food Prot. 79, 337–344. https://doi.org/10.4315/0362-028X.JFP-15-387

Gebretsadik, S., Kassa, T., Alemayehu, H., Huruy, K., Kebede, N., 2011. Isolation and characterization of *Listeria monocytogenes* and other *Listeria* species in foods of animal origin in Addis Ababa, Ethiopia. J. Infect. Public Health 4, 22–29. https://doi.org/10.1016/J.JIPH.2010.10.002

Gianfranceschi, M., Gattuso, A., Tartaro, S., Aureli, P., 2002. Incidence of *Listeria monocytogenes* in food and environmental samples in Italy between 1990 and 1999: Serotype distribution in food, environmental and clinical samples. Eur. J. Epidemiol. 18, 1001–1006. https://doi.org/10.1023/A:1025849532417

Glaser, P., Frangeul, L., Buchrieser, C., Rusniok, C., Amend, A., Baquero, F., Berche, P., Bloecker, H., Brandt, P., Chakraborty, T., Charbit, A., Chetouani, F., Couvé, E., De Daruvar, A., Dehoux, P., Domann, E., Domínguez-Bernal, G., Duchaud, E., Durant, L., Dussurget, O., Entian, K.D., Fsihi, H., Garcia-Del Portillo, F., Garrido, P., Gautier, L., Goebel, W., Gómez-López, N., Hain, T., Hauf, J., Jackson, D., Jones, L.M., Kaerst, U., Kreft, J., Kuhn, M., Kunst, F., Kurapkat, G., Madueño, E., Maitournam, A., Mata Vicente, J., Ng, E., Nedjari, H., Nordsiek, G., Novella, S., De Pablos, B., Pérez-Diaz, J.C., Purcell, R., Remmel, B., Rose, M., Schlueter, T., Simoes, N., Tierrez, A., Vázquez-Boland, J.A., Voss, H., Wehland, J., Cossart, P., 2001. Comparative genomics of *Listeria* species. Science. 294, 849–852. https://doi.org/10.1126/science.1063447

Goldfine, H., Shen, H. (Eds.), 2007. *Listeria monocytogenes*: Pathogenesis and Host Response. Springer, New York, New York.

Guillet, C., Join-Lambert, O., Le Monnier, A., Leclercq, A., Mechaï, F., Mamzer-Bruneel, M.-F., Bielecka, M.K., Scortti, M., Disson, O., Berche, P., Vazquez-Boland, J., Lortholary, O., Lecuit, M., 2010. Human listeriosis caused by *Listeria ivanovii*. Emerg. Infect. Dis. 16, 136–138. https://doi.org/10.3201/eid1601.091155

Heisick, J.E., Wagner, D.E., Nierman, M.L., Peeler, J.T., 1989. *Listeria* spp. found on fresh market produce. Appl. Environ. Microbiol. 55, 1925–1927.

Henriksen, S.D., 1978. Chapter I Serotyping of bacteria. Methods Microbiol. 12, 1–13. https://doi.org/10.1016/S0580-9517(08)70355-6

Ho, J.L., Shands, K.N., Friedland, G., Eckind, P., Fraser, D.W., 1986. An outbreak of type 4b *Listeria monocytogenes* infection involving patients from eight Boston hospitals. Arch. Intern. Med. 146. https://doi.org/10.1001/archinte.1986.00360150134016\

Hoelzer, K., Sauders, B.D., Sanchez, M.D., Olsen, P.T., Pickett, M.M., Mangione, K.J., Rice, D.H., Corby, J., Stich, S., Fortes, E.D., Roof, S.E., Grohn, Y.T., Wiedmann, M., Oliver, H.F., 2011. Prevalence, distribution, and diversity of *Listeria monocytogenes* in retail environments, focusing on small establishments and establishments with a history of failed inspections. J. Food Prot. 74, 1083–1095. https://doi.org/10.4315/0362-028X.JFP-10-567

Hof, H., Nichterlein, T., Kretschmar, M., 1997. Management of listeriosis. Clin. Microbiol. Rev. 10, 345–357.

Jordan, K., Fox, E.M., Wagner, M. (Eds.), 2014. *Listeria monocytogenes*: Methods and Protocols. Springer, New York, New York.

Jorgensen, J., Waite-Cusic, J., Kovacevic, J., 2019a. Diversity of *Listeria* spp. in Produce Handling and Processing Facilities in the Pacific Northwest. Submitt. to Food Microbiol.

Jorgensen, J., Waite-Cusic, J., Kovacevic, J., 2019b. Prevalence of *Listeria* spp. in Produce Handling and Processing Facilities in the Pacific Northwest.

Kathariou, S., 2002. *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. J. Food Prot. 65, 1811–1829. https://doi.org/10.4315/0362-028X-65.11.1811

Kells, J., Gilmour, A., 2004. Incidence of *Listeria monocytogenes* in two milk processing environments, and assessment of *Listeria monocytogenes* blood agar for isolation. Int. J. Food Microbiol. 91, 167–174. https://doi.org/10.1016/S0168-1605(03)00378-7

Kovacevic, J., Arguedas-Villa, C., Wozniak, A., Tasara, T., Allen, K.J., 2013a. Examination of food chain-derived *Listeria monocytogenes* strains of different serotypes reveals considerable diversity in inlA genotypes, mutability, and adaptation to cold temperatures. Appl. Environ. Microbiol. 79, 1915–1922. https://doi.org/10.1128/AEM.03341-12

Kovacevic, J., Bohaychuk, V.M., Barrios, P.R., Gensler, G.E., Rolheiser, D.L., McMullen, L.M., 2009. Evaluation of environmental sampling methods and rapid detection assays for recovery and identification of *Listeria* spp. from meat processing facilities. J. Food Prot. 72, 696–701. https://doi.org/10.4315/0362-028X-72.4.696

Kovacevic, J., Sagert, J., Wozniak, A., Gilmour, M.W., Allen, K.J., 2013b. Antimicrobial resistance and co-selection phenomenon in *Listeria* spp. recovered from food and food production environments. Food Microbiol. 34, 319–327. https://doi.org/10.1016/J.FM.2013.01.002

Lakicevic, B., Nastasijevic, I., Dimitrijevic, M., 2017. Whole genome sequencing: an efficient approach to ensuring food safety. IOP Conf. Ser. Earth Environ. Sci. 85. https://doi.org/10.1088/1755-1315/85/1/012052

Leong, D., Alvarez-Ordõnez, A., Jordan, K., 2014. Monitoring occurrence and persistence of *Listeria monocytogenes* in foods and food processing environments in the Republic of Ireland. Front. Microbiol. 436, 1-8. https://doi.org/10.3389/fmicb.2014.00436

Leong, D., Nicaogáin, K., Luque-Sastre, L., Mcmanamon, O., Hunt, K., Alvarez-Ordóñez, A., Scollard, J., Schmalenberger, A., Fanning, S., O'byrne, C., Jordan, K., 2017. A 3-year multi-food study of the presence and persistence of *Listeria monocytogenes* in 54 small food businesses in Ireland. Int. J. Food Microbiol. 249, 18–26. https://doi.org/10.1016/j.ijfoodmicro.2017.02.015

Liao, J., Wiedmann, M., Kovac, J., 2017. Genetic stability and evolution of the sigB allele, used for *Listeria* sensu stricto subtyping and phylogenetic inference. Appl. Environ. Microbiol. 83. https://doi.org/10.1128/AEM.00306-17

Linke, K., Rückerl, I., Brugger, K., Karpiskova, R., Walland, J., Muri-Klinger, S., Tichy, A., Wagner, M., Stessl, B., 2014. Reservoirs of *Listeria* species in three environmental ecosystems. Appl. Environ. Microbiol. 80, 5583–5592. https://doi.org/10.1128/AEM.01018-14

Little, C., Taylor, F., Sagoo, S., Gillespie, I., Grant, K., McLauchlin, J., 2007. Prevalence and level of *Listeria monocytogenes* and other *Listeria* species in retail pre-packaged mixed vegetable salads in the UK. Food Microbiol. 24, 711–717. https://doi.org/10.1016/j.fm.2007.03.009

Liu, D., 2006. Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. J. Med. Microbiol. 55, 645–659. https://doi.org/10.1099/jmm.0.46495-0

McCollum, J.T., Cronquist, A.B., Silk, B.J., Jackson, K.A., O'Connor, K.A., Cosgrove, S., Gossack, J.P., Parachini, S.S., Jain, N.S., Ettestad, P., Ibraheem, M., Cantu, V., Joshi, M., DuVernoy, T., Fogg, N.W., Gorny, J.R., Mogen, K.M., Spires, C., Teitell, P., Joseph, L.A., Tarr, C.L., Imanishi, M., Neil, K.P., Tauxe, R. V., Mahon, B.E., 2013. Multistate outbreak of listeriosis associated with cantaloupe. N. Engl. J. Med. 369, 944–953. https://doi.org/10.1056/NEJMoa1215837

Miettinen, M.K., Björkroth, K.J., Korkeala, H.J., 1999a. Characterization of *Listeria monocytogenes* from an ice cream plant by serotyping and pulsed-field gel electrophoresis. Int. J. Food Microbiol. 46, 187–192.

Miettinen, M.K., Siitonen, A., Heiskanen, P., Haajanen, H., Björkroth, K.J., Korkeala, H.J., 1999b. Molecular epidemiology of an outbreak of febrile gastroenteritis caused by *Listeria monocytogenes* in cold-smoked rainbow trout. J. Clin. Microbiol. 37, 2358–2360.

Milillo, M.S., 2015. Resistance to food processing sanitizers and heavy metals in *Listeria monocytogenes* from British Columbia, Canada and antibiogram profiles of clinically relevant Listeria monocytogenes from British Columbia and Alberta, Canada. University of British Columbia. https://doi.org/10.14288/1.0165806

Muhterem-Uyar, M., Dalmasso, M., Sorin Bolocan, A., Hernandez, M., Kapetanakou, A.E., Kuchta, T., Manios, S.G., Melero, B., Minarovi cov, J., Ioana Nicolau, A., Rovira, J., Skandamis, P.N., Jordan, K., Rodríguez-L azaro, D., Stessl, B., Wagner, M., 2015. Environmental sampling for *Listeria monocytogenes* control in food processing facilities reveals three contamination scenarios. Food Control 51, 94–107. https://doi.org/10.1016/j.foodcont.2014.10.042

Núñez-Montero, K., Leclercq, A., Moura, A., Vales, G., Peraza, J., Pizarro-Cerdá, J., Lecuit, M., 2018. *Listeria* costaricensis sp. nov. Int. J. Syst. Evol. Microbiol. 68, 844–850. https://doi.org/10.1099/ijsem.0.002596

Olaimat, A.N., Al-Holy, M.A., Shahbaz, H.M., Al-Nabulsi, A.A., Abu Ghoush, M.H., Osaili, T.M., Ayyash, M.M., Holley, R.A., 2018. Emergence of antibiotic resistance in *Listeria monocytogenes* isolated from food products: a comprehensive review. Compr. Rev. Food Sci. Food Saf. 17, 1277–1292. https://doi.org/10.1111/1541-4337.12387

Orsi, R.H., Bakker, H.C. de., Wiedmann, M., 2011. *Listeria monocytogenes* lineages: genomics, evolution, ecology, and phenotypic characteristics. Int. J. Med. Microbiol. 301, 79–96. https://doi.org/10.1016/j.ijmm.2010.05.002

Orsi, R.H., Wiedmann, M., 2016. Characteristics and distribution of *Listeria* spp., including Listeria species newly described since 2009. Appl. Microbiol. Biotechnol. 100, 5273–5287. https://doi.org/10.1007/s00253-016-7552-2

Painter, J.A., Hoekstra, R.M., Ayers, T., Tauxe, R. V., Braden, C.R., Angulo, F.J., Griffin, P.M., 2013. Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998–2008. Emerg. Infect. Dis. 19, 407–415. https://doi.org/10.3201/eid1903.111866

Petran, R.L., Zottou, E.A., Gravani, R.B., 1988. Incidence of *Listeria monocytogenes* in market samples of fresh and frozen vegetables. J. Food Sci. 53, 1238–1240. https://doi.org/10.1111/j.1365-2621.1988.tb13576.x

Piffaretti, J.C., Kressebuch, H., Aeschbacher, M., Bille, J., Bannerman, E., Musser, J.M., Selander, R.K., Rocourt, J., 1989. Genetic characterization of clones of the bacterium *Listeria monocytogenes* causing epidemic disease. Proc. Natl. Acad. Sci. 86, 3818–3822. https://doi.org/10.1073/pnas.86.10.3818

Prazak, A.M., Murano, E.A., Mercado, I., Acuff, G.R., 2002. Prevalence of *Listeria monocytogenes* during production and postharvest processing of cabbage. J. Food Prot. 65, 1728–1734. https://doi.org/10.4315/0362-028x-65.11.1728

Rasmussen, O.F., Skouboe, P., Dons, L., Rossen, L., Olsen, J.E., 1995. *Listeria monocytogenes* exists in at least three evolutionary lines: evidence from flagellin, invasive associated protein and listeriolysin O genes. Microbiology 141, 2053–2061. https://doi.org/10.1099/13500872-141-9-2053

Revazishvili, T., Kotetishvili, M., Stine, O.C., Kreger, A.S., Morris, J.G., Sulakvelidze, A., 2004. Comparative analysis of multilocus sequence typing and pulsed-field gel electrophoresis for characterizing *Listeria monocytogenes* strains isolated from environmental and clinical sources. J. Clin. Microbiol. 42, 276–285. https://doi.org/10.1128/jcm.42.1.276-285.2004

Ribera, L.A., Palma, M.A., Paggi, M., Knutson, R., Masabni, J.G., Anciso, J., 2012. Economic analysis of food safety compliance costs and foodborne illness outbreaks in the United States. Horttechnology 22, 150–156. https://doi.org/10.21273/HORTTECH.22.2.150

Rota, C., Yanguela, J., Blanco, D., Carraminana, J.J., Arino, A., Herrera, A., 1996. High prevalence of multiple resistance to antibiotics in 144 *Listeria* isolates from Spanish dairy and meat products. J. Food Prot. 59, 938–943. https://doi.org/10.4315/0362-028X-59.9.938

Salcedo, C., Arreaza, L., Alcalá, B., de la Fuente, L., Vázquez, J.A., 2003. Development of a multilocus sequence typing method for analysis of *Listeria monocytogenes* clones. J. Clin. Microbiol. 41, 757–762. https://doi.org/10.1128/JCM.41.2.757-762.2003

Sauders, B.D., Sanchez, M.D., Rice, D.H., Corby, J., Stich, S., Fortes, E.D., Roof, S.E., Wiedmann, M., 2009. Prevalence and molecular diversity of *Listeria monocytogenes* in retail establishments. J. Food Prot. 72, 2337–2349. https://doi.org/10.4315/0362-028X-72.11.2337

Scallan, E., Hoekstra, R.M., Angulo, F.J., Tauxe, R. V, Widdowson, M.-A., Roy, S.L., Jones, J.L., Griffin, P.M., 2011. Foodborne illness acquired in the United States--major pathogens. Emerg. Infect. Dis. 17, 7–15. https://doi.org/10.3201/eid1701.p11101

Schlech, W.F., Lavigne, P.M., Bortolussi, R.A., Allen, A.C., Haldane, E.V., Wort, A.J., Hightower, A.W., Johnson, S.E., King, S.H., Nicholls, E.S., Broome, C. V., 1983. Epidemic listeriosis — evidence for transmission by food. N. Engl. J. Med. 308, 203–206. https://doi.org/10.1056/NEJM198301273080407

Schönberg, A., Bannerman, E., Courtieu, A.L., Kiss, R., McLauchlin, J., Shah, S., Wilhelms, D., 1996. Serotyping of 80 strains from the WHO multicentre international typing study of *Listeria monocytogenes*. Int. J. Food Microbiol. 32, 279–87.

Seeliger, H.P.R., Höhne, K., 1979. Chapter II serotyping of *Listeria monocytogenes* and related species. Methods Microbiol. 13, 31–49. https://doi.org/10.1016/S0580-9517(08)70372-6

Sheng, L., Edwards, K., Tsai, H.-C., Hanrahan, I., Zhu, M.-J., 2017. Fate of *Listeria monocytogenes* on fresh apples under different storage temperatures. Front. Microbiol. 8. https://doi.org/10.3389/fmicb.2017.01396

Sheng, L., Hanrahan, I., Sun, X., Taylor, M.H., Mendoza, M., Zhu, M.-J., 2018. Survival of *Listeria innocua* on Fuji apples under commercial cold storage with or without low dose continuous ozone gaseous. Food Microbiol. 76, 21–28. https://doi.org/10.1016/j.fm.2018.04.006

Smith, J.L., 1998. Foodborne illness in the elderly. J. Food Prot. 61, 1229–1239.

Snapir, Y.M., Vaisbein, E., Nassar, F., 2006. Low virulence but potentially fatal outcome—*Listeria ivanovii*. Eur. J. Intern. Med. 17, 286–287. https://doi.org/10.1016/J.EJIM.2005.12.006

Strawn, L.K., Gröhn, Y.T., Warchocki, S., Worobo, R.W., Bihn, E.A., Wiedmann, M., 2013. Risk factors associated with *Salmonella* and *Listeria monocytogenes* contamination of produce fields. Appl. Environ. Microbiol. 79, 7618–7627. https://doi.org/10.1128/AEM.02831-13

Swaminathan, B., Barrett, T.J., Hunter, S.B., Tauxe, R.V., Force, C.P.T., 2001. PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. Emerg. Infect. Dis. 7, 382–389. https://doi.org/10.3201/eid0703.010303

Swaminathan, B., Gerner-Smidt, P., 2007. The epidemiology of human listeriosis. Microbes Infect. 9, 1236–1243. https://doi.org/10.1016/j.micinf.2007.05.011

Tan, X., Chung, T., Chen, Y., Macarisin, D., LaBorde, L., Kovac, J., 2019. The occurrence of *Listeria monocytogenes* is associated with built environment microbiota in three tree fruit processing facilities. Microbiome 115, 1-8. https://doi.org/10.1186/s40168-019-0726-2

Tappero, J.W., Schuchat, A., Deaver, K.A., Mascola, L., Wenger, J.D., 1995. Reduction in the incidence of human listeriosis in the United States. JAMA 273, 1118–1122. https://doi.org/10.1001/jama.1995.03520380054035

Temple, M.E., Nahata, M.C., 2000. Treatment of listeriosis. Ann. Pharmacother. 34, 656–661. https://doi.org/10.1345/aph.19315

Tompkin, R.B., 2002. Control of *Listeria monocytogenes* in the food-processing environment. J. Food Prot. 65, 709–725. https://doi.org/10.4315/0362-028X-65.4.709

Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J., Kreft, J., 2001. *Listeria* pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. 14, 584–640. https://doi.org/10.1128/CMR.14.3.584-640.2001

Vivant, A.-L., Garmyn, D., Piveteau, P., 2013. *Listeria monocytogenes*, a down-to-earth pathogen. Front. Cell. Infect. Microbiol. 3, 87. https://doi.org/10.3389/fcimb.2013.00087

Walsh, D., Duffy, G., Sheridan, J.J., Blair, I.S., McDowell, D.A., 2001. Antibiotic resistance among *Listeria*, including *Listeria monocytogenes*, in retail foods. J. Appl. Microbiol. 90, 517–522. https://doi.org/10.1046/j.1365-2672.2001.01273.x

Ward, T.J., Ducey, T.F., Usgaard, T., Dunn, K.A., Bielawski, J.P., 2008. Multilocus genotyping assays for single nucleotide polymorphism-based subtyping of *Listeria monocytogenes* isolates. Appl. Environ. Microbiol. 74, 7629–7642. https://doi.org/10.1128/AEM.01127-08

Weis, J., Seeliger, H.P., 1975. Incidence of *Listeria monocytogenes* in nature. Appl. Microbiol. 30, 29–32.

Welshimer, H.J., 1960. Survival of *Listeria monocytogenes* in soil. J. Bacteriol. 80, 316–320.

Wenger, J.D., Swaminathan, B., Hayes, P.S., Green, S.S., Pratt, M., Pinner, R.W., Schuchat, A., Broome, C. V., 1990. *Listeria monocytogenes* contamination of turkey franks: evaluation of a production facility. J. Food Prot. 53, 1015–1019. https://doi.org/10.4315/0362-028X-53.12.1015

Wilson, I.G., 1995. Occurrence of *Listeria* species in ready to eat foods. Epidemiol. Infect. 115, 519–526. https://doi.org/10.1017/s0950268800058684

Zhang, G., Brown, E.W., González-Escalona, N., 2011. Comparison of real-time PCR, reverse transcriptase real-time PCR, loop-mediated isothermal amplification, and the FDA conventional microbiological method for the detection of *Salmonella* spp. in produce. Appl. Environ. Microbiol. 77, 6495–6501. https://doi.org/10.1128/AEM.00520-11

Zhu, Q., Gooneratne, R., Hussain, M.A., 2017. *Listeria monocytogenes* in fresh produce: outbreaks, prevalence and contamination levels. Foods 6, 1–11. https://doi.org/10.3390/foods6030021

Appendices

Appendix A

Oregon State University

DO YOU GROW, HARVEST, PACKAGE OR PROCESS PRODUCE IN THE PACIFIC NORTHWEST?

(Oregon, Washington & Idaho)

ARE YOU INTERESTED IN PARTICIPATING IN FOOD SAFETY RESEARCH?

Oregon State University's Food Science and Technology Department and the Food Innovation Center are looking for produce farms, packinghouses, or processing facilities to participate in a project that will investigate sources and control of *Listeria* in the Pacific Northwest (PNW).

Project includes:

- Environmental swabbing and sampling for *Listeria* spp. and *Listeria monocytogenes* on non-food contact surfaces to better understand the prevalence in produce handling environments and potential contamination sources.
  - **Double blinding the samples so they cannot be traced to your farm/facility is an option.**
- **Education and training on:**
  - New *Listeria* guidelines and requirements under FSMA
  - Sources of *Listeria* contamination and preventive controls
  - Developing environmental mentoring programs using hygienic zones approach
  - Hands-on training for environmental sample collection

THERE IS NO COST TO YOU TO PARTICIPATE IN THE PROJECT! PARTICIPATION WOULD INCLUDE:

- **Year One (2018):**
  - Two environment swabbing sessions
- **Year Two (2019):**
  - One environment swabbing session
  - Optional participation in a workshop

WHAT WE HOPE TO ACCOMPLISH DURING THIS PROJECT AND PROVIDE PNW PRODUCE INDUSTRY WITH:

- Region specific data on *Listeria* prevalence and contamination points
- Information on new guidelines, and practices and factors important in controlling *Listeria* in produce operations
- Hands-on training for setting up environmental monitoring strategies, and sample collection

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Appendix B

Produce Handling and Processing Facility Survey

1. How many different produce products go through this facility?

    a. Do you handle, pack or process these products (i.e. what is your main production technique)?
        i. Do any of your products receive a "kill step"?
            - If so, what type of processing/kill step does it go through?
            - Do all of your products receive a kill step or is any product sold as fresh product, without any processing?
        ii. Are you a registered processing facility, with the FDA under section 415 of the Federal Food, Drug, and Cosmetic Act (Preventive Controls Rule)?

    b. Can you give a list of the products that have been handled, packed or processed through your facility within the last year?
        i. You mentioned XXX commodities – were they all handled/packed etc. in the last year, or if not, which ones were?

2. How many permanent employees do you have?

    a. During peak periods, do you get extra help?
        i. If so, on average, how many employees do you have during those peak periods?

    b. How many employees do you have during slow periods?

3. Do you have written SSOPs (sanitation standard operating procedures)?

    a. Can you, step by step, explain your SOPs?
        i. How long do you process before cleaning?
        ii. How do you clean your food contact surfaces, such as conveyor belts, bins?
        iii. How do you clean your facility environment not in contact with produce, such as your floors and drains, forklifts?
        iv. Do you follow-up cleaning with sanitizer treatment?
        v. If so, do you do this for your food and non-food contact surfaces?
        vi. Who does the cleaning and sanitation in your facility?
        vii. Do you have a separate crew that performs these duties or do these individuals do other activities in the facility?
        viii. How often do you clean and sanitize your food contact surfaces?

Produce Handling and Processing Facility Survey Continued

4. What sanitizer(s) do you use in your facilities?

- **a.** Do you rotate your sanitizer(s)?
- **b.** Do you follow manufacturers recommendations?
  - **i.** Do you know what those recommendations are?
  - **ii.** Would you share that information?

5. Do you have an environmental monitoring program?

- **a.** How are you using your EMP program (i.e. as a verification of preventive controls)?
  - **i.** What is the main purpose of your EMP program (i.e., why do you do it)?
    - Is it a requirement as part of FSMA, such as a verification step for preventive controls?
    - Is it required from your buyers?
    - Is it an operational step to confirm that your cleaning and sanitation is being done correctly?
- **b.** What is your sampling frequency – do you take some samples daily, or is it a weekly, or monthly schedule?
- **c.** Do you have a set number of samples that you take each time, or does it vary?
  - **i.** How many samples do you take (per day/month/year)?
- **d.** What do you use to collect samples (i.e. sponges or cotton swabs)
  - **i.** From what company?
- **e.** Where do you sample (what zones, non-food contact or food contact)?
  - **i.** Does the sampling frequency change depending on whether samples are collected from food contact or non-food contact surfaces (e.g., in the summer more samples, or more FCS etc.)?
- **f.** Do you test in house or send out for testing?
  - **i.** What do you test for (e.g., *Salmonella*, *Listeria*)?
    - ATPs?
    - Indicator organisms? Which?
    - Pathogens? Which?

Appendix C

SAMPLE TRACKING FORM
Pathogen Testing in Produce Handling and Processing Facilities

Facility code:	**Sampling date:**	**Sampling collection begin time:**
Collected by:		**Sampling round:**
Sample collection end time:		**Lab use:**

Environmental Samples		
Type of surface	**Sampling site – Description (Place mark on facility map)**	**SAMPLE NAME**
❒ Non-food contact	1	
❒ Non-food contact	2	
❒ Non-food contact	3	
❒ Non-food contact	4	
❒ Non-food contact	5	
❒ Non-food contact	6	
❒ Non-food contact	7	

Appendix D

Table D1. List of *Listeria* spp. strains collection from produce handling and processing facilities from May 2018 to April 2019, in the States of Oregon and Washington. Up to three isolates were taken from each positive sampling site, and serogrouping was done only for *L. monocytogenes* isolate.

Strain ID	Species	Serogroup	Facility ID	Sample Site Description	Sample ID
WRLP388	*L. innocua*		A	Drain	247
WRLP389	*L. innocua*		A	Drain	247
WRLP390	*L. innocua*		A	Drain	247
WRLP391	*L. innocua*		A	Drain	249
WRLP392	*L. innocua*		A	Drain	249
WRLP393	*L. innocua*		A	Drain	249
WRLP394	*L. monocytogenes*	4b 4d or 4e	A	Drain	252
WRLP395	*L. monocytogenes*	4b 4d or 4e	A	Drain	252
WRLP396	*L. monocytogenes*	4b 4d or 4e	A	Drain	252
WRLP422	*L. monocytogenes*	4b 4d or 4e	A	Drain	425
WRLP423	*L. welshimeri*		A	Drain	425
WRLP424	*L. welshimeri*		A	Drain	425
WRLP425	*L. welshimeri*		A	Drain	426
WRLP426	*L. welshimeri*		A	Drain	426
WRLP427	*L. welshimeri*		A	Drain	426
WRLP428	*L. welshimeri*		A	Drain	428
WRLP429	*L. welshimeri*		A	Drain	428
WRLP430	*L. welshimeri*		A	Drain	428
WRLP431	*L. ivanovii*		A	Drain	429
WRLP432	*L. ivanovii*		A	Drain	429
WRLP433	*L. ivanovii*		A	Drain	429
WRLP434	*L. monocytogenes*	1/2a or 3a	A	Drain	434
WRLP435	*L. monocytogenes*	1/2a or 3a	A	Drain	434
WRLP436	*L. monocytogenes*	1/2a or 3a	A	Drain	434
WRLP437	*L. innocua*		A	Floor	440
WRLP438	*L. innocua*		A	Floor	440
WRLP439	*L. innocua*		A	Floor	440
WRLP440	*L. monocytogenes*	4b 4d or 4e	A	Entry point	441
WRLP441	*L. monocytogenes*	4b 4d or 4e	A	Entry point	441
WRLP442	*L. monocytogenes*	4b 4d or 4e	A	Entry point	441
WRLP443	*L. monocytogenes*	4b 4d or 4e	A	Other	447
WRLP444	*L. welshimeri*		A	Other	447
WRLP445	*L. welshimeri*		A	Other	447
WRLP446	*L. monocytogenes*	4b 4d or 4e	A	Other	448
WRLP447	*L. monocytogenes*	4b 4d or 4e	A	Other	448
WRLP448	*L. monocytogenes*	4b 4d or 4e	A	Other	448
WRLP449	*L. welshimeri*		A	Other	449
WRLP450	*L. welshimeri*		A	Other	449
WRLP451	*L. welshimeri*		A	Other	449
WRLP452	*L. welshimeri*		A	Drain	459
WRLP453	*L. welshimeri*		A	Drain	459

Strain ID	Species	Serogroup	Facility ID	Sample Site Description	Sample ID
WRLP454	*L. welshimeri*		A	Drain	459
WRLP455	*L. monocytogenes*	4b 4d or 4e	A	Drain	460
WRLP456	*L. monocytogenes*	4b 4d or 4e	A	Drain	460
WRLP457	*L. monocytogenes*	4b 4d or 4e	A	Drain	460
WRLP458	*L. monocytogenes*	4b 4d or 4e	A	Drain	464
WRLP459	*L. monocytogenes*	4b 4d or 4e	A	Drain	464
WRLP460	*L. monocytogenes*	4b 4d or 4e	A	Drain	464
WRLP461	*L. monocytogenes*	4b 4d or 4e	A	Drain	466
WRLP462	*L. monocytogenes*	4b 4d or 4e	A	Drain	466
WRLP463	*L. monocytogenes*	4b 4d or 4e	A	Drain	466
WRLP464	*L. monocytogenes*	4b 4d or 4e	A	Drain	467
WRLP465	*L. monocytogenes*	4b 4d or 4e	A	Drain	467
WRLP466	*L. monocytogenes*	4b 4d or 4e	A	Drain	467
WRLP467	*L. monocytogenes*	4b 4d or 4e	A	Drain	468
WRLP468	*L. monocytogenes*	4b 4d or 4e	A	Drain	468
WRLP469	*L. monocytogenes*	4b 4d or 4e	A	Floor	480
WRLP470	*L. monocytogenes*	4b 4d or 4e	A	Floor	480
WRLP471	*L. monocytogenes*	4b 4d or 4e	A	Floor	480
WRLP472	*L. monocytogenes*	1/2a or 3a	A	Portable item	483
WRLP473	*L. monocytogenes*	1/2a or 3a	A	Portable item	483
WRLP474	*L. welshimeri*		A	Portable item	484
WRLP475	*L. welshimeri*		A	Portable item	484
WRLP476	*L. welshimeri*		A	Portable item	484
WRLP477	*L. monocytogenes*	1/2a or 3a	A	Forklift	490
WRLP478	*L. monocytogenes*	1/2a or 3a	A	Forklift	490
WRLP479	*L. monocytogenes*	1/2a or 3a	A	Forklift	490
WRLP480	*L. welshimeri*		A	Entry point	493
WRLP481	*L. welshimeri*		A	Entry point	493
WRLP482	*L. welshimeri*		A	Entry point	493
WRLP483	*L. monocytogenes*	4b 4d or 4e	A	Entry point	494
WRLP484	*L. monocytogenes*	4b 4d or 4e	A	Entry point	494
WRLP485	*L. innocua*		A	Outside sample	498
WRLP486	*L. monocytogenes*	4b 4d or 4e	A	Outside sample	498
WRLP487	*L. monocytogenes*	4b 4d or 4e	A	Outside sample	498
WRLP488	*L. monocytogenes*	1/2a or 3a	A	Outside sample	499
WRLP489	*L. monocytogenes*	1/2a or 3a	A	Outside sample	499
WRLP490	*L. monocytogenes*	1/2a or 3a	A	Outside sample	499
WRLP491	*L. monocytogenes*	1/2a or 3a	A	Outside sample	500
WRLP492	*L. monocytogenes*	4b 4d or 4e	A	Outside sample	500
WRLP493	*L. monocytogenes*	4b 4d or 4e	A	Outside sample	500
WRLP494	*L. monocytogenes*	4b 4d or 4e	A	Cooler	504
WRLP495	*L. monocytogenes*	4b 4d or 4e	A	Drain	508
WRLP496	*L. monocytogenes*	4b 4d or 4e	A	Drain	508
WRLP497	*L. monocytogenes*	4b 4d or 4e	A	Drain	508
WRLP498	*L. monocytogenes*	1/2a or 3a	A	Drain	510
WRLP499	*L. monocytogenes*	4b 4d or 4e	A	Drain	511
WRLP500	*L. monocytogenes*	4b 4d or 4e	A	Drain	511

Strain ID	Species	Serogroup	Facility ID	Sample Site Description	Sample ID
WRLP501	*L. monocytogenes*	4b 4d or 4e	A	Drain	511
WRLP502	*L. monocytogenes*	4b 4d or 4e	A	Drain	516
WRLP503	*L. monocytogenes*	4b 4d or 4e	A	Drain	516
WRLP504	*L. monocytogenes*	4b 4d or 4e	A	Drain	516
WRLP505	*L. innocua*		A	Drain	517
WRLP506	*L. innocua*		A	Drain	517
WRLP507	*L. innocua*		A	Drain	517
WRLP508	*L. monocytogenes*	1/2a or 3a	A	Portable item	521
WRLP509	*L. innocua*		A	Portable item	521
WRLP510	*L. innocua*		A	Portable item	521
WRLP511	*L. monocytogenes*	4b 4d or 4e	A	Portable item	524
WRLP512	*L. monocytogenes*	4b 4d or 4e	A	Portable item	524
WRLP513	*L. monocytogenes*	4b 4d or 4e	A	Portable item	524
WRLP514	*L. innocua*		A	Entry point	526
WRLP515	*L. innocua*		A	Entry point	526
WRLP516	*L. innocua*		A	Entry point	526
WRLP517	*L. monocytogenes*	4b 4d or 4e	A	Entry point	527
WRLP518	*L. innocua*		A	Entry point	527
WRLP519	*L. monocytogenes*	4b 4d or 4e	A	Outside sample	528
WRLP520	*L. monocytogenes*	4b 4d or 4e	A	Outside sample	528
WRLP521	*L. monocytogenes*	4b 4d or 4e	A	Outside sample	529
WRLP522	*L. monocytogenes*	1/2a or 3a	A	Outside sample	529
WRLP523	*L. monocytogenes*	4b 4d or 4e	A	Outside sample	529
WRLP524	*L. monocytogenes*	1/2a or 3a	A	Outside sample	530
WRLP525	*L. monocytogenes*	1/2a or 3a	A	Outside sample	530
WRLP526	*L. monocytogenes*	1/2a or 3a	A	Outside sample	530
WRLP527	*L. monocytogenes*	4b 4d or 4e	A	Cooler	531
WRLP528	*L. monocytogenes*	4b 4d or 4e	A	Cooler	531
WRLP529	*L. monocytogenes*	4b 4d or 4e	A	Cooler	531
WRLP530	*L. monocytogenes*	4b 4d or 4e	A	Other	553
WRLP531	*L. monocytogenes*	4b 4d or 4e	A	Other	553
WRLP532	*L. monocytogenes*	4b 4d or 4e	A	Other	553
WRLP533	*L. monocytogenes*	4b 4d or 4e	A	Other	554
WRLP534	*L. monocytogenes*	4b 4d or 4e	A	Other	554
WRLP535	*L. monocytogenes*	4b 4d or 4e	A	Other	554
WRLP354	*L. monocytogenes*	4b 4d or 4e	C	Drain	27
WRLP355	*L. monocytogenes*	4b 4d or 4e	C	Drain	27
WRLP356	*L. monocytogenes*	4b 4d or 4e	C	Drain	27
WRLP377	*L. monocytogenes*	4b 4d or 4e	C	Floor	185
WRLP378	*L. monocytogenes*	4b 4d or 4e	C	Floor	185
WRLP380	*L. monocytogenes*	4b 4d or 4e	C	Drain	187
WRLP381	*L. monocytogenes*	4b 4d or 4e	C	Drain	187
WRLP382	*L. monocytogenes*	4b 4d or 4e	C	Entry point	212
WRLP383	*L. monocytogenes*	4b 4d or 4e	C	Entry point	212
WRLP384	*L. monocytogenes*	4b 4d or 4e	C	Entry point	212
WRLP386	*L. monocytogenes*	4b 4d or 4e	C	Forklift	213
WRLP387	*L. monocytogenes*	4b 4d or 4e	C	Forklift	213

Strain ID	Species	Serogroup	Facility ID	Sample Site Description	Sample ID
WRLP407	*L. monocytogenes*	4b 4d or 4e	D	Drain	279
WRLP408	*L. monocytogenes*	4b 4d or 4e	D	Drain	279
WRLP409	*L. monocytogenes*	4b 4d or 4e	D	Drain	279
WRLP357	*L. innocua*		E	Drain	88
WRLP358	*L. innocua*		E	Drain	88
WRLP359	*L. innocua*		E	Drain	88
WRLP360	*L. monocytogenes*	1/2a or 3a	E	Equipment Leg	104
WRLP361	*L. monocytogenes*	1/2a or 3a	E	Equipment Leg	104
WRLP362	*L. monocytogenes*	1/2a or 3a	E	Equipment Leg	104
WRLP373	*L. innocua*		E	Drain	161
WRLP374	*L. innocua*		E	Drain	161
WRLP375	*L. innocua*		E	Drain	161
WRLP367	*L. monocytogenes*	1/2a or 3a	G	Drain	138
WRLP368	*L. monocytogenes*	1/2a or 3a	G	Drain	138
WRLP369	*L. monocytogenes*	1/2a or 3a	G	Drain	138
WRLP370	*L. monocytogenes*	1/2a or 3a	G	Entry point	141
WRLP371	*L. monocytogenes*	1/2a or 3a	G	Entry point	141
WRLP372	*L. monocytogenes*	1/2a or 3a	G	Entry point	141
WRLP410	*L. monocytogenes*	1/2a or 3a	G	Floor	337
WRLP411	*L. monocytogenes*	1/2a or 3a	G	Floor	337
WRLP412	*L. monocytogenes*	1/2a or 3a	G	Floor	337
WRLP413	*L. innocua*		G	Outside sample	347
WRLP414	*L. innocua*		G	Outside sample	347
WRLP415	*L. innocua*		G	Outside sample	347
WRLP416	*L. monocytogenes*	1/2a or 3a	G	Forklift	384
WRLP417	*L. monocytogenes*	1/2a or 3a	G	Forklift	384
WRLP418	*L. innocua*		G	Forklift	384
WRLP419	*L. innocua*		G	Outside sample	386
WRLP420	*L. innocua*		G	Outside sample	386
WRLP421	*L. innocua*		G	Outside sample	386

Appendix E

Oregon State University

Pathogen Environmental Monitoring Workshop for Pacific Northwest Food Industries

(Oregon, Washington & Idaho)

Food processing, handling and storage facilities can create conditions that allow microorganisms to become established and thrive. Some of these microorganisms have the potential to cause foodborne illness if they contaminate food products.

Identifying niche locations and hotspots that house these microorganisms through both routine and investigative environmental programs is critical for producers of ready-to-eat foods and raw agricultural commodities.

This **two-day** workshop is intended to provide you with knowledge and best practices on developing and implementing an effective Pathogen Environmental Monitoring (PEM) program.

What to expect?

- *Through hands-on activities you will:*
  - *Become familiar with environmental foodborne pathogens,*
  - *Learn how to collect swabs,*
  - *Identify issues through case studies from the industry,*
  - *Get tips on how to design and establish a PEM program,*
  - *Have opportunity to discuss effective controls and corrective action steps in small working groups.*
- *You will receive:*
  - *All course materials,*
  - *Certificate of course attendance,*
  - *Meals (lunch and refreshments).*

WHEN AND WHERE?

- **Dates and times:**
  Day 1: June 12, 2019 8:00 AM to 5:00 PM
  Day 2: June 13, 2019 8:00 AM to 3:00 PM
- **Location:**
  Food Innovation Center
  1207 NW Naito Parkway, Portland, OR 97209

REGISTRATION:

- **Register through Eventbrite**
  https://fic-pem-june.eventbrite.com
- **Cost:**
  $150 per person,
  $125 for multiple registrations.

This workshop is subsidized through OR SCBGP grant ODA-5010-GR.

Please note:
Registrations are limited. NO substitutions, transfers, or refunds will be issued within one week of the training start date.

Questions about registration and additional PEM workshops this Summer and Fall?

Contact Catherine Haye at catherine.haye@oregonstate.edu or by calling 503.872.6680.

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Oregon State University
Food Innovation Center

Oregon State University
Food Innovation Center

Pathogen Environmental Monitoring Workshop
June 12-13, 2019, Portland OR
Food Innovation Center
AGENDA

Day 1	Modules and activities	Instructors
8:00 AM	Registration, welcome, pre-test & introductions	Jovana
8:45 AM	Module 1: Intro to foodborne pathogens in food industry environments	Jovana
9:30 AM	Module 2: FSMA and pathogen environmental monitoring	John
10:00 AM	***Break***	
10:15 AM	Module 3: Environmental sampling plan, methods, and procedures	Joy
	Exercise	
12:15 PM	***Lunch***	
1:00 PM	Swabbing activity	John
1:30 PM	Module 4: Sanitary design - facility and equipment	Dave
	Exercise	
2:45 PM	Module 5: Cleaning and sanitation	Dave
3:30 PM	***Break***	
3:45 PM	Exercise	
4:15 PM	Module 6: What happens when you get positives?	Jovana
5:00 PM	***Adjourn***	

Day 2	Modules and activities	Instructors
8:00 AM	Recap and Module 6 continuation	Jovana
	Exercise	
9:00 AM	Case study 1	John
	Exercise	
10:30 AM	***Break***	
10:45 AM	Module 7: Corrective actions	Joy
12:00 PM	***Lunch***	
1:00 PM	Exercise	Joy
2:00 PM	Closing, post-test, evaluations	Jovana
3:00 PM	***Adjourn***	

Appendix F

Processing Samples for *Salmonella* spp.

After incubation for 24 hours ± 2 hours at 35°C ± 2°C in Buffered Peptone Water (BPW, Neogen, Lansing, MI, USA) *Salmonella* samples were transferred to Tetrathionate Broth (TT, Neogen, Lansing, MI, USA) and Rappaport-Vassiliadis medium (RV, Neogen, Lansing, MI, USA). 1 mL of BPW was transferred to 10 mL of TT and 0.1 mL of BPW was transferred to RV, samples were vortexed for 15 seconds. RV samples were incubated for 24 hours ± 2 hours at 42°C ± 0.2°C (circulating, thermostatically controlled, water bath) and TT samples were incubated for 24 hours ± 2 hours at 35°C ± 2°C. After 24 hours ± 2 hours samples were vortexed for 15 seconds and streaked onto Hektoen Enteric Agar (HE, Neogen, Lansing, MI, USA) with a 10 µL loop, and incubated 24 hours ± 2 hours at 35°C ± 2°C. After 24 hours ± 2 hours, and if available, eight to ten typical or atypical colonies from each sample from TT and RV were picked and stabbed from HE on to CHROMagar™ *Salmonella* Plus (CHROMagar, Paris, France).

Typical *Salmonella* colonies on HE appear blue-green to blue with or without black centers. Typical *Salmonella* colonies may produce colonies with large, glossy black centers or may appear as almost completely black colonies. Atypical *Salmonella* colonies may look yellow with or without a black center. CHROMagar plates were incubated for 18 to 24 hours at 35°C ± 2°C. *Salmonella* colonies, typical and atypical, appear mauve on CHROMagar. If confirmed on CHROMagar, 4-8 colonies were confirmed by RT-qPCR according to the Zhang et al. *Salmonella inv*A assay, modified to be SYBR green assay. If confirmed by RT-qPCR, colonies from CHROMagar were separately picked and streaked onto isolate BAPs and incubated for 24 hours ± 2 hours at 35°C ± 2°C.

Processing Samples for *Salmonella* – Confirmation RT-qPCR *invA* assay

After CHROMagar™ *Salmonella* Plus confirmation (CHROMagar, Paris, France), colonies moved on to RT-qPCR detection of *Salmonella* (Zhang et al., 2011). DNA extraction was done with a Lucigen QuickExtract™ DNA kit (Lucigen, Middleton, WI, USA) according to the manufacturer's instructions. A single colony, corresponding to each sample and sample location, was picked from the CHROMagar plates and put into 0.5 mL of Lucigen QuickExtract Solution™. A control strain, *Salmonella typhimurium* ATCC 700720, was run a long side all potential samples, also having been picked from CHROMagar plates. The samples were then vortexed for 15 seconds, heated for 6 minutes at 65°C, vortexed again for 15 seconds, heated for 2 minutes at 98°C, and then vortexed for 15 seconds.

The primers for this assay, targeting the *invA* gene, were purchased from Integrated DNA Technologies (IDT, Coralville, IA, USA) and are given in Table 1. All RT-qPCR runs were done using the Fast SYBR® Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) and run on an Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific, Waltham, MA, USA). The Fast SYBR® Green Master Mix contains all elements used for the assay, and were used according to manufacture recommendations. The final concentration of the *invA* gene in the RT-qPCR mix was 200nM. The qPCR parameters were as follows: initial incubation for 120 seconds at 50°C, 120 seconds at 95°C to activate the polymerase, 40 cycles of denaturation for 15 seconds at 95°C and then primer annealing and extension for 30 seconds at 60°C, with a melt curve ran on the back end.

Table F1. *Salmonella* spp. *invA* gene primers for RT-qPCR identification.

Target	Primer	Sequence (5'->3')	Product size (bp)
*inv*A	invA_176F	CAACGTTTCCTGCGGTACTGT	116
	invA_291R	CCCGAACGTGGCGATAATT	