THESIS

LISTERIA MONOCYTOGENES AND OTHER *LISTERIA* SPECIES IN SMALL AND VERY SMALL READY-TO-EAT MEAT PROCESSING PLANTS

Submitted by

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ABSTRACT OF THESIS

DETECTION AND MOLECULAR CHARACTERIZATION OF *LISTERIA MONOCYTOGENES* AND OTHER *LISTERIA* SPECIES IN THE PROCESSING PLANT ENVIRONMENT

Listeria monocytogenes is the causative agent of listeriosis, a severe foodborne disease associated with a high case fatality rate. To prevent product contamination with *L. monocytogenes,* it is crucial to understand *Listeria* contamination patterns in the food processing plant environment. The aim of this study was to monitor *Listeria* contamination patterns for two years in six small or very small ready-to-eat (RTE) meat processing plants using a routine combined cultural and molecular typing program. Each of the six plants enrolled in the study were visited on a bi-monthly basis for a two-year period where samples were collected, microbiologically analyzed for *Listeria* and isolates from positive samples were characterized by molecular subtyping. Year one of the project focused only on non-food contact environmental samples within each plant, and year two focused again on non-food contact environmental samples as well as food contact surfaces and finished RTE meat product samples from participating plants. Between year one and year two of sampling, we conducted an in-plant training session

involving all employees at each plant. During this training session, we informed employees about general *Listeria* knowledge such as ecology, transmission and control strategies. Also, we informed each plant of the testing and molecular subtyping results obtained in the first year of the study. Employees also were given a pre- and post-training evaluation, which included 23 questions on *Listeria*, to probe knowledge gained through the training session. A common characteristic among almost all plants enrolled in our study was the persistence of a single or few predominant *L. monocytogenes* and/or other *Listeria* spp. molecular subtype(s) in the plant environment. Identification of persistent strains and their associated harborage sites in the environment of each plant highlight the continued need for adequate cleaning and sanitation practices to eliminate harborage sites and reduce the risk of transmission to the finished product. Interestingly, we not only observed a significant increase in plant employee knowledge regarding *Listeria* following the in-plant training sessions, but we also detected a significant decrease in *Listeria* contamination across all six plants when comparing testing results from year 1 and 2. With combined molecular detection and subtyping, we were able to help increase plant awareness about *Listeria* contamination patterns, identify harborage sites and intervention strategies to better control *Listeria* in the plant environment.

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DEDICATION

I would like to dedicate this thesis to my parents, Wayne and Billie Williams. Thank you for your unconditional support throughout my studies. I feel very blessed and honored to have you as my parents. Thank you for giving me the chance to prove and improve myself through all my walks of life. Thank you for believing in me and teaching me the importance of hard work and dedication by setting an example. Without your support and involvement in my life it would never have been possible for me to make it this far. You guy's have always been there for me even when your own health and lives should have been the number one importance. I'm not sure what I would ever do without you two and I love you both with all my heart. Thanks for being the wonderful parents that you are, and I will forever look up to you guys and never forget how amazing you have made my life. Thank you so much for everything, and I hope you know that I never could have accomplished this without you.

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CHAPTER I

LITERATURE REVIEW

LISTERIA IN THE PROCESSING PLANT ENVIRONMENT

1.1. *Listeria* Genus and Taxonomy

The genus *Listeria* includes Gram-positive, non-sporeforming, catalase positive rod shaped bacteria, which were once classified into the family Corynebacteriaceae. It was named *Bacterium monocytogenes* by Murray et al. (1926), whom isolated a 1–2 μm long and 0.5 μm wide round-ended Gram-positive rod in dead laboratory rabbits and guinea-pigs in Cambridge, United Kingdom (Farber and Peterkin, 1991). Following unusual deaths of gerbils in South Africa in the late 1920s, this bacterium was named *Listerella hepatolytica* by Pirie in honor of Lord Joseph Lister who determined that in order to prevent infections, surgeons need to sterilize their instruments before each operation (Ryser, 1999a). Because the strains isolated by Murray et al. (1926) and Pirie (1927) showed great similarity, the bacterium was renamed *Listerella monocytogenes.* However, the generic name *Listerella* had previously been used for a protozoa and in 1940, Pirie thus proposed changing the name to *Listeria monocytogenes.* This name was accepted, even though the genus name already existed in botanical taxonomy, including an orchid named *Listeria,* and in zoology, including a diptera called *Listeria* (Seeliger, 1961). Genera of *Listeria* and *Brochothrix* are members of the family

Listeriaceae, the order *Bacillales,* the class *Bacilli* and the phylum *Firmicutes* (Ludwig et al., 2009).

Currently, it is widely accepted that the core phylogeny of *Listeria* consists of six different species: *L. monocytogenes, L. innocua, L. seeligeri, L. welshimeri, L. grayii* and *L. ivanovii* (Wiedmann, 2002). A seventh species, *Listeria murrayi,* was previously recognized in the *Listeria* genus; however, DNA-DNA hybridization analysis, multiolocus enzyme electrophoresis, and rRNA restriction fragment length polymorphism analysis, proved that *L. murrayi* appeared to be subspecies within *L. grayii*. (Boerlin et al. 1991, 1992; Rocourt et al. 1992). In addition, recent studies described the occurrence of atypical hemolytic *L. innocua* strains that carry the *Listeria* pathogenicity island I (Graves et al., 2009; Johnson et al., 2004). Most recently, studies have proposed recognition of two novel species within the *Listeria* genus, including *Listeria marthii* and *Listeria rocourtiae* (Graves et al., 2009; Leclercq et al., *in press*).

Listeria species appear as small rods ranging in size from 0.4 to 0.5 by 1-2µm, and sometimes are found to be arranged in short chains when viewed under the microscope. A coccoid appearance may be seen in direct smears. *Listeria* produces flagella at room temperature and exhibit a tumbling motion when examined in broth and a swarming motility can be observed in semi-soft agar at 30^{o}C (Roberts et al., 2009), but flagella are not produced at 37°C (Peel et al., 1988).

1.2. *Listeria* in Nature

All *Listeria* species are ubiquitous in nature and the bacterium often is described to demonstrate a plant saprophyte lifestyle. *Listeria* commonly is detected in soil (Weis et al., 1975), water (Watkins et al., 1981), manure, sewage (Colburn et al., 1990; Watkins et al., 1981), vegetation (Weis et al., 1975), animal feed (Wiedmann et al., 1996), and farm environments (Fenlon et al., 1996; Nightingale et al., 2004). *L. monocytogenes* has also been isolated from at least 42 species of wild and domestic mammals and 17 avian species, including domestic and game fowl as well as crustaceans, fish, oysters, ticks, and flies (Schuchat et al., 1992). Also, this pathogen may be carried in the intestinal tracts of a small percentage of the human population without apparent symptoms (Rouquette et al, 1996; Grif et al., 2003).

Studies aimed at isolating *Listeria* in natural environments not associated with domestic livestock indicated that other *Listeria* spp. were detected at higher frequencies than *L. monocytogenes* (MacGowan et al., 1994). This study specifically reported a high incidence of *L. seeligeri* in samples collected from the general environment. In another study where samples of grass, leaves, stems, and roots were sampled, *L. monocytogenes* was detected in nine of 10 samples of wilting grass; however, no *L. monocytogenes* was isolated from samples taken of the roots or stems (Fenlon et al., 1996). *Listeria* has also been shown to be common and present in surface samples from natural water sources, such as lakes, rivers, and streams (Dijkstra et al., 1982). Next, animal feeds, and improperly fermented ensiled feeds in particular, have been associated with listeriosis outbreaks in sheep and cattle (Fenlon et al., 1986; Gitter et al., 1986). The contaminated

silage has been the outcome of poor silage quality such as inadequate moisture content, which may facilitate the survival and growth of *L. monocytogenes* (Grønstøl et al., 1979).

The wide distribution of *L. monocytogenes* in nature allows this bacterium to be easily spread and cause infection. *Listeria monocytogenes* can cause infection by several transmission routes such as ingestion of contaminated foods (e.g. unpasteurized milk or contaminated ready-to-eat foods; Schlech et al. 1983, Fleming et al. 1985, Linnan et al. 1988), transmission of the organism from mother to fetus in utero (McLauchlin, 1990), directly to the fetus at the time of birth, or by direct contact with the organism which can cause lesions on the skin (McLauchlin 1990). The ability of *L. monocytogenes* to survive and multiply in many non-host habitats and host species, and the number of possible transmission routes, makes this pathogen difficult to control in its natural environment.

1.3. *L. monocytogenes* in Food

Contamination of foods by *L. monocytogenes* can occur at any point in the food chain, including on farms, in food processing plants, in retail establishments and in the home (Saunders, 2006, Nightingale, 2005, Lappi, 2004). *L. monocytogenes* can be detected in a wide range of foods, including both raw and processed foods. Many foods such as soft cheeses, hot dogs, and seafood have been implicated in listeriosis outbreaks, but *L. monocytogenes* also can be isolated from other foods such as beef, pork, fermented sausages, fresh produce and fish products (Rocourt and Cossart, 1997). *Listeria* has been shown to survive within cultured buttermilk, butter and yogurt; of which these specific foods primarily depend on adequate fermentation to yield a low product pH that does not support *Listeria* growth. Many studies have shown that a wide variety of meats can

become contaminated with *L. monocytogenes* and most contamination is observed on meat product and poultry. For example, Bailey et al. (1990) reported that between 12-60% of raw chicken was contaminated with *L. monocytogenes* and young birds were colonized by this human pathogen at a higher rate. Many studies have shown that the ability of *L. monocytogenes* to survive and grow on meat is dependent on temperature, pH of the meat, type of tissue, and initial miroflora already present on the meat's surface (Farber and Peterkin, 1991).

Since *Listeria monocytogenes* is found in soil and water, raw vegetables can become contaminated from the soil or from manure used as fertilizer (Schlech et al., 1983). Animals (i.e., wildlife and domestic livestock) can be asymptomatic carriers of *L. monocytogenes* and contaminate foods of animal origin such as dairy and meats through asymptomatic shedding in milk and feces. Not only can *L. monocytogenes* be isolated from raw foods (e.g. vegetables, uncooked meats), but it also can be detected in processed foods such as soft cheeses and delicatessen meats (Seeliger, 1961, Fenlon et al. 1996, Fenlon, 1999). Although *Listeria* can easily be inactivated by cooking and pasteurization (Petran and Zottola 1989), it remains a significant problem in ready-to-eat foods (e.g., frankfurters and delicatessen meats) that may become cross-contaminated by exposure in the food processing plant environment after cooking but before packaging (Tompkin, 2002).

1.4. *Listeria* in the Food Processing Plant Environment

One key reason that *Listeria* presents such a problem for many food manufacturers is simply because food processing conditions and the associated

processing environment permit the growth of *Listeria*. This Gram-positive, facultative anaerobe, intracellular rod is capable of growth in a broad range of temperatures including refrigeration (e.g. 1°C to 45°C), wide range of pH conditions (e.g. 4.3-9.5), relatively low water activity ($\geq$ 0.90), and high salt concentrations (up to 10%), enabling survival and growth in many different food and food-associated environments (Farber and Peterkin, 1991). Many studies have demonstrated the ability of *L. monocytogenes* to colonize, multiply, and persist in the food processing environment as well as on food processing equipment over extended periods, showing the environmental survival characteristics of this foodborne pathogen (Lappi et al., 2004, Kabuki et al., 2004). Overall, *Listeria* is a very adaptable pathogen that is capable of survival even after freezing, surface dehydration, and spray chilling; however, *Listeria* can easily be killed with proper cooking (Seeliger and Jones 1986, Junttila et al. 1988).

1.5. Pathogenic *Listeria monocytogenes* and Disease

In humans, most listeriosis cases are observed in neonates, the elderly, pregnant women, or otherwise immunocompromised individuals such as those on chemotherapy or immuno-suppressant drugs usually transmitted through the consumption of contaminated foods (Mead et al., 1999). On very rare occasions, the pathogen also can be transmitted directly from infected animals to humans; which has been observed in veterinarians, farmers, and abattoir personnel handling contaminated tissues (Posfay-Barbe et al., 2009). Vertical transmission from mother to neonate can occur transplacentally or the infant can become infected during delivery through contact with organisms in the birth canal (Posfay-Barbe et al., 2009). Schuchat et al (1991) described an unusual example of

Listeria transmission in a nosocomial outbreak involving neonates, whom became infected through contact with contaminated mineral oil that was being used to bathe the infants within a specific neonatal unit.

1.6. Symptoms of the Disease

Pregnant women are the most at-risk population for contracting a *Listeria monocytogenes* infection, and they are about 20 times more likely than other healthy adults to become ill with listeriosis. If pregnant women acquire listeriosis, the fetus is most heavily infected, leading to spontaneous abortion, stillbirths, or sepsis in infancy. About one-third of *Listeria* cases represent pregnancy-associated cases (Cossart and Bierne, 2001). In most cases, the fetus or newborn is more likely than the mother to be affected by listeriosis associated with pregnancy (Silver, 1998); the perinatal and neonatal mortality rate is 80 percent (FDA/CFSAN, 2003). The Mayo Clinic found the following symptoms of listeriosis to be common in infants who contract this disease: loss of appetite, lethargy, jaundice, vomiting, skin rash, and/or breathing difficulties (Mayo Clinic, 2009).

Listeriosis can develop as two different forms of disease, a non-invasive form known as listerial gastroenteritis, or a severe invasive form of disease that often is accompanied by severe clinical manifestations. The non-invasive form of listeriosis results in a wide variety of symptoms ranging from fever, muscle aches, and gastrointestinal symptoms such as nausea or diarrhea. Five days to three weeks after ingestion of the bacterium, *Listeria* can infect deeper tissues leading to an invasive form of listeriosis causing a systemic infection (FDA/CFSAN, 2003). If the infection spreads to the nervous system,

symptoms such as headache, stiff neck, loss of balance, confusion, or convulsions can occur. With brain involvement, listeriosis may mimic a stroke, and lead to meningitis or encephalitis (Crum, 2002). Other at-risk individuals for contracting listeriosis include people with weakened or compromised immune systems, cancer patients, transplant recipients, diabetics, and persons with AIDS (Schuchat et al., 1992). Dietary precautions should be taken by those individuals most at risk of acquiring a *L. monocytogenes* infection in order to help decrease the chances of acquiring severe systemic disease.

1.7. Listeriosis Cases, Hospitalizations and Deaths

Annual projections in the United States indicate that approximately 2,500 cases of human listeriosis will occur where nearly 500 of these cases progress to death and 300 cases will require hospitalization (Mead et al., 1999). This projection may be underestimated by half due to asymptomatic symptoms occurring in healthy individuals who become infected, but show no clinical signs (Mead et al., 1999). The "Healthy People 2010" initiative was established by the federal government to establish achieve a 50% reduction in the overall number of listeriosis cases by 2010 (USDA-FSIS, 2003). This national health promotion would involve national, state, local, government agencies, voluntary, nonprofit, communities, and individuals together to lead in a fight to improve the health of the Americans (USDA-FSIS, 2003). A noticeable decrease in listeriosis was observed between 1996-2001, but reached a plateau after 2002 (CDC, 2009).

1.8. Outbreaks and Sporadic Cases, or Small Clusters

L. monocytogenes has been implicated in multiple large outbreaks worldwide. Each year in the United States, the incidence of listeriosis is estimated to range from 3.4 per million to 4.4 cases per million (CDC, 2003; Tappero et al., 1995). Internationally, the incidence of listeriosis varies from 3.5 persons per million in Bristol, England up to 6 to 7 million persons per million in Denmark (Slutsker and Schuchat, 1999). Outbreaks of listeriosis have been associated with consumption of raw or contaminated milk, contaminated vegetables, and RTE meats such as hot dogs or pate'. Most listeriosis infections are sporadic or occur in small clusters and identifying the food vehicle responsible for sporadic cases or small clusters is very difficult and the food attribution of listeriosis remains to be fully elucidated (Slutsker and Schuchat, 1999). When a group, or cluster of infections occurs, epidemiological investigations are performed in order to prevent further illness as well as to identify the responsible food vehicle and food processing plant where that food was produced. An outbreak of foodborne illness may be identified if a cluster of two or more illnesses are linked to the same or similar strain of a given foodborne pathogen and the same food source (CDC, 2006). Identification of small clusters is important and should be investigated in order to identify the possible common source responsible for the outbreak as well as preventing further illnesses. Fortunately, human listeriosis is rare; however, this disease has a very long incubation period ranging from 7-60 days making it difficult to attribute illness to a specific food product (Farber and Peterkin, 1991).

Between 1970 and 2002, 12 identified severe listeriosis outbreaks occurred in the United States. These 12 listeriosis outbreaks that occurred between 1970-2002 in the United States, caused a total of 466 listeriosis infections, where two of these twelve

outbreaks involved more than 100 cases each (USDA-FSIS, 2003). Raw eggs were implicated in one of the outbreaks, and the remaining eleven outbreaks were associated with consumption of RTE products. The largest reported outbreak, involving 142 cases, was linked to consumption of contaminated Mexican-style soft cheese (Linnan et al., 1988). Consumption of frankfurters and deli meats was responsible for the second largest listeriosis outbreaks causing disease in 101 individuals and leading to 21 deaths (CDC, 1998; CDC, 1999; Mead et al., 1999). During this outbreak, Mead et al. (2006) found these two RTE products were from the same manufacturing establishment, and that this specific plant was being renovated at the time; therefore, this construction quite possibly could have led to post-processing contamination of the RTE product (Mead et al., 2006). Other known foods implicated in domestic listeriosis outbreaks are contaminated dairy products, which have led to four known outbreaks, meat which has been implicated in three known outbreaks, and eggs and vegetables have each caused a single outbreak (USDA-FSIS, 2003). With better surveillance, awareness of listeriosis and molecular subtyping, responsible food vehicles are more easily identified and traced back to a specific brand or plant. Therefore, the specific food vehicles found to be implicated in outbreaks within the United States included pasteurized milk, Mexican-style cheese, butter, eggs, deli turkey meat, pâté, and vegetables (USDA-FSIS, 2003).

In countries other than the United States, 18 outbreaks involving >1,000 listeriosis cases occurred between 1970 and 2000, where a single food vehicle was identified in 17 of the outbreaks. Consumption of contaminated dairy products, specifically butter and cheese, were implicated in six outbreaks, meat products such as pâté, pork tongue, and RTE ham caused five outbreaks, seafood (e.g. smoked mussels, cold-smoked trout, raw

fish) were implicated in four outbreaks, and raw vegetables and cabbage caused two outbreaks (Lyytikainen et al., 2000; Jensen, 1994; Ryser, 1999a; Jacquet et al., 1995; Goulet et al., 1995; Mitchell, 1991; Misrachi et al., 1991; Brett et al., 1998; McLaughlin et al., 1991; Schlech et al., 1983; Bille, 1990; Lennon et al., 1984; Le Souef and Walters, 1981). In one specific outbreak in 1978 in Austria, multiple food vehicles such as unpasteurized milk and vegetables were found to be responsible for the listeriosis outbreak (Allerberger and Guggenbichler, 1989). The most common foods implicated in all listeriosis cases are dairy and RTE meat products such as frankfurters, deli meat, pâté and pork tongue. The most common dairy products implicated in outbreaks outside the United States were soft cheeses and mold-ripened cheeses (USDA-FSIS, 2003). These findings are similar to those found from case-control studies of sporadic listeriosis cases, in which un-reheated frankfurters, undercooked chicken, soft cheeses and foods purchased at a deli counter were associated with listeriosis (Schwartz et al., 1988; Schuchat et al., 1992). These results also were consistent with a study by Pinner et al. (1992), who found that the foods most likely to cause listeriosis were RTE foods, and foods from which serotype 4b was isolated. Serotype 4b has been found in almost 70% of worldwide outbreaks (US FDA/CFSCAN & USDA/FSIS & CDC., 2003). Listeriosis outbreaks occuring domestically and internationally have been shown to be similar in which the *L. monocytogenes* strains causing outbreaks have a higher frequency of being serotype 4b than any other serotype. Also, the specific food groups which are implicated in causing infection as well as the case fatality rates appear to be similar when comparing worldwide outbreaks of listeriosis. (Mead et al., 1999).

1.9. Virulence and Intracellular Life Cycle

As a pathogen, *L. monocytogenes* infects a wide range of host species and is capable of passively or actively entering many host cell types. The primary route of infection is across the intestinal epithelium after consumption of contaminated food products by the host. Following entry into the bloodstream, most of the bacteria end up in the liver and spleen by way of macrophages. Unless replication is controlled by an effective host innate immune response, the bacteria escape from immune clearance and continue to divide and replicate. Host survival then depends on the development of an effective adaptive immune response; otherwise, the bacteria re-enter the bloodstream to cause potentially fatal systemic or central nervous system infections. The ability of *L. monocytogenes* to replicate in the cytosol of infected host cells and to spread from cell to cell enables it to avoid humoral immune responses (Pamer, 2004). A number of bacterial surface proteins, including the internalins InlA and InlB, have been shown to contribute to bacterial invasion of host cells (Seveau et al., 2007). InlA binds E-cadherin, a host cell adhesion molecule, whereas InlB binds to the hepatocyte growth factor receptor, Met; binding to these receptors enables *L. monocytogenes* to gain entry into host cells through the use of the host endocytic machinery (Pizarro-Cerda et al., 2006). Once internalized, *L. monocytogenes* mediates its escape from the membrane-bound vacuole by secreting a pore-forming cytolysin, known as listeriolysin O (LLO), and two phospholipases, which work together to break down the phagosome in which it resides (Schnupf et al., 2007; Kathariou et al., 1987). Within the host cell cytosol, the bacteria replicate using nutrients that are acquired from the host. *L. monocytogenes* then moves through the cell and into adjacent cells using actin polymerization as a motility force, which it directs through its surface protein actin assembly-inducing protein (ActA)

(Pizarro-Cerda et al., 2006). The bacteria enter adjacent cells and secrete LLO and the broad-specificity phosphatidylcholine phospholipase C (PC-PLC) to escape from the double-membraned secondary vacuoles that are formed as a result of cell-to-cell spread (Schnupf et al., 2007; Kathariou et al., 1987).

1.10. *L. monocytogenes* Subtyping Methods

Many different subtyping methods have been used for the differentiation of *L. monocytogenes* such as conventional phenotypic methods and DNA-based subtyping methods. Conventional and phenotypic subtyping methods include serotyping, phage typing, and multilocus enzyme electrophoresis (Wiedmann, 2002). Serotyping is based on the antigens expressed on the bacterial cell surface, which are detected by antisera. Serotyping has been a classical tool in subtyping of *L. monocytogenes* and is based on somatic (O) and flagellar (H) antigens. *L. monocytogenes* strains are divided into 13 serotypes: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e and 7 (Seeliger and Höhne 1979). However, over 95% of strains isolated from human cases and foods belong to serotypes 1/2a, 1/2b and 4b, therefore limiting the usefulness of serotyping in both epidemiological and contamination investigations (Farber and Peterkin 1991). Phage typing characterizes the *L. monocytogenes* isolates by the susceptibility to lysis by a set of phages. This method is very rapid and high throughput. Phage typing is an efficient method for large scale subtyping of *L. monocytogenes* (Audurier and Martin 1989). A standardized procedure and phage set have been developed and assembled (Rocourt et al. 1985, McLauchlin et al. 1996). Overall this method has been shown to be highly discriminatory; however, the high number of non-typeable strains, specifically,

serotype 1/2 strains is a major disadvantage of the method (Rocourt et al. 1985). Moreover, the method is only available at a limited number of reference laboratories because of the need to maintain stocks of biologically active phages and control strains. Lastly, multilocus enzyme electrophoresis (MEE) uses different constitutive enzymes that are separated by electrophoresis and banding patterns are analyzed (Wiedmann, 2002).

Band-based molecular subtyping methods commonly used to characterize *L. monocytogenes* isolates include random amplification of polymorphic DNA (RAPD), pulsed-field gel electrophoresis, and *Eco*RI ribotyping. These methods are more discriminatory and have a high level of reproducibility and standardization across laboratories. Pulsed-field gel electrophoresis digests complete bacterial DNA with one or more rare cutting restriction enzyme(s) (i.e., ApaI, AscI) creating 10-25 large fragments ranging in size from 10kb to 800 kb, which then are separated by size by a hexagonal gel electrophoresis apparatus (Römling et al. 1994). PFGE typing has been widely used to characterize *L. monocytogenes* isolates and standardized protocols have been developed and implemented for routine typing purposes (Brosch et al. 1991, Buchrieser et al. 1991, 1993, Jacquet et al. 1995, Brosch et al. 1996, Destro et al. 1996, Unnerstad et al. 1996, Giovannacci et al. 1999, Dauphin et al. 2001, Graves and Swaminathan 2001, Vela et al. 2001). PFGE typing is considered to be the current gold standard for typing of *L. monocytogenes* due to its high discriminatory ability and reproducibility (Wiedmann, 2002). Therefore, national networks for storing and comparing PFGE fingerprints are used in many countries including the United States (Graves and Swaminathan 2001, Rantala et al. 2001). Next, *Eco*RI ribotyping is an automated method based on the use of nucleic acid probes targeting ribosomal genes after restriction enzyme analysis of

chromosomal DNA (Grimont and Grimont 1986). Since ribosomal RNA genes are highly conserved in all bacteria and include both conserved and variable regions, this method commonly is used for identification and subtyping purposes (Farber, 1996). Ribotyping begins by digestion of bacterial DNA by a frequent cutting restriction enzyme creating 300-500 smaller DNA fragments that are then separated by gel electrophoresis. The separated DNA fragments are transferred onto membrane and hybridized with labeled probes containing 23S and 16S sequences (Grimont and Grimont 1986). Ribotyping has been used to characterize *L. monocytogenes* in taxonomical and epidemiological investigations (Baloga and Harlander 1991, Graves et al. 1991, Arimi et al. 1997, Dalton et al. 1997, Wiedmann et al. 1997, Allerberger and Fritschel 1999, Gendel and Ulaszek 2000, de Cesare et al. 2001, Nadon et al. 2001, Suihko et al. 2002). Ribotyping has many advantages such as typeability and reproducibility as well as availability of an automated platform and a large database of well-characterized isolates. However, disadvantages of this method include high cost of equipment and reagents as well as the lack of discriminating power, specifically for serotype 4b isolates, which can render this an insufficient method for some epidemiological investigations (Swaminathan et al. 1996).

Although DNA band-based methods most frequently have been employed to characterize *L. monocytogenes* isolates in molecular epidemiology investigations, DNA sequenced based subtyping methods also have been developed to characterize *L. monocytogenes* isolates (Wiedmann, 2002). DNA sequencing-based subtyping involves sequencing of multiple genes or gene fragments to differentiate bacterial subtypes and determine relatedness between isolates. For example, multilocus sequence typing

(MLST), which involves sequencing of multiple housekeeping genes and/or sequencing multiple virulence genes, can be used as a DNA sequence-based subtyping method to characterize bacteria populations (Maiden et al. 1998, Enright and Spratt 1999). Most commonly, MLST schemes involve sequencing 450–500 bp long fragments of five to seven housekeeping genes. For each gene, sequences are aligned and polymorphisms are used to group isolates into unique allelic types. Unique combinations of allelic types across different genes are used to group isolates into multi-locus sequence types. MLST is expensive and time consuming, but a World-Wide Web site has been developed for a global MLST database, which is one huge advantage to this method (Chan et al. 2001a).

1.11. Genetic Diversity and Epidemiological Association Between Strains/Lineages and Disease

L. monocytogenes strains are grouped in three major genetic lineages (lineages I, II, and III), with lineage I containing isolates belonging to serotypes 1/2b, 3b, 3c, and 4b, lineage II containing isolates belonging to serotypes 1/2a, 1/2c, and 3a, and lineage III containing isolates belonging serotypes 4a and 4c. (Weidmann et al., 1997). Lineage I includes the major epidemic clone strains implicated in multiple listeriosis epidemics worldwide; lineage II isolates have been isolated from human clinical cases but are overrepresented among foods; and lineage III isolates are mostly found in ruminants (Kathariou et al., 2002). Previous molecular subtyping studies identified four major epidemic clones (EC) of *L. monocytogenes* (i.e., ECI-IV; Kathariou, 2003). Among these epidemic clones, ECI, a serotype 4b cluster, was linked to major outbreaks in different countries, including coleslaw (Nova Scotia, 1981), soft cheese (Switzerland, 1983 to 1987, and California, 1985), and pork tongue (France, 1992) outbreaks (Roberts et al.,

2009). ECII was first observed in the 1998-1999 U.S. multistate outbreak associated with hot dogs and then again in the 2002 U.S. multistate listeriosis outbreak associated with contaminated turkey deli meat (Kathariou et al., 2002). ECIV, another serotype 4b cluster, caused an outbreak linked to pâté (United Kingdom, 1988) and another outbreak linked to vegetables (Boston, 1983) (McLaughlin et al., 1991; Fleming et al., 1985). ECIII isolates belong to serotype 1/2a and were linked to a 1988 sporadic listeriosis case and the multi-state turkey deli meat outbreak that occurred in United States during 2000, where RTE meat products associated with both disease incidence were produced at the same facility (CDC, 2000). Saunders et al. (2006) suggested that concurrent sporadic listeriosis cases were also caused by EC strains, showing that between 1998 and 2002 two large temporal clusters of sporadic case isolates throughout Michigan, Ohio and New York shared the same ribotype as ECII; therefore, indicating that listeriosis outbreaks and sporadic clusters may be more commonly linked than previously thought.

1.12. Use of Molecular Subtyping Methods to Identify Persistent Strains, Harborage Sites, and Determine Transmission in the Processing Plant Environment

Molecular subtyping methods are useful in the identification of harborage sites and transmission patterns within a processing plant environment. DNA-based subtyping methods generate banding patterns that can be used to identify harborage sites where *Listeria* persists and elucidate transmission patterns of *Listeria* in the processing plant environment (Lappi et al., 2004). Ribotyping has been used extensively in characterization of *L. monocytogenes,* epidemiological investigations, and determination of contamination routes (Baloga and Harlander, 1991, Graves et al. 1991, Arimi et al. 1997, Dalton et al. 1997, Wiedmann et al. 1997, Allerberger and Fritschel 1999, Gendel

and Ulaszek 2000, de Cesare et al. 2001, Nadon et al. 2001, Suihko et al. 2002). Sampling sites or product, combined with characterization of bacterial isolates by molecular subtyping techniques, has proven to be an effective approach to trace both pathogen and spoilage contamination (Destro et al. 1996, Nesbakken et al. 1996). By comparison of isolates recovered at different stages of processing, on equipment, in the air, and in the final product, it is possible to define the specific sources and sites of product contamination. These molecular approaches have been used in contamination studies including other various pathogens, such as *Bacillus cereus*, *Escherichia coli* and *Yersinia enterocolitica;* and a number of methods have been used such as multilocus enzyme electrophoresis, plasmid profiling, analysis of fatty acid profiles, ribotyping, RAPD, AFLP and PFGE typing (Dykes et al. 1993, Destro et al. 1996, Nesbakken et al. 1996, Björkroth and Korkeala 1997, Lin et al. 1998, Raleya et al. 1998, Fredriksson-Ahomaa et al. 2000, Georgnaras et al. 2001). These subtyping methods are crucial in the food industry and have had a positive impact in outbreak surveillance of this pathogen as well as a better understanding of the transmission, ecology, and evolution of *L. monocytogenes.*

1.13. *Listeria* Final Rule, Three Production Alternatives and Sampling Plans

In November 2002, the U.S. Food Safety Inspection Service (FISIS) enforced an intensified testing program for all RTE processing plants that produce at-risk RTE products (defined below) without a validated testing system for *L. monocytogenes*. Plants that had a validated testing program, but chose not to share their testing data with FSIS on an ongoing basis, also were subject to the intensified testing program. These

provisions were incorporated into the interim final rule (9 CFR Part 430, 2003). The purpose of this rule is to ensure that processors of at-risk RTE products take one or more specific steps to prevent *L. monocytogenes* contamination of their products. These steps range from focused sanitation steps, to adding formulation or processing steps designed to kill or inhibit *L. monocytogenes* (9 CFR Part 430, 2003).

Most plants producing RTE products that are exposed to the environment after cooking will be required to do some laboratory testing of food contact surfaces associated with RTE product handling and packaging. The amount of testing required is to be determined by the type of RTE product, how it is formulated, processed and marketed. For many of the smaller-scale plants, it is recommended that at least one food contact surface be tested for *L. monocytogenes* or other *Listeria* species per month. In most situations, the processor will be required to perform *L. monocytogenes* testing of food contact surfaces in the environment in which RTE products are handled after cooking.

Specifically, under 9 CFR Part 430, the USDA/FSIS requires establishments that produce fully cooked RTE meat and poultry products that are (1) exposed to the environment after lethality treatments and (2) support the growth of *L. monocytogenes*, to have in their Hazard Analysis Critical Control Points (HACCP) plan, sanitation Standard Operating Procedures (SOPs), or other prerequisite programs, controls that prevent product adulteration by *L. monocytogenes*. The interim final rule, became effective October 6, 2003 and mandates incorporation of one of the three alternative approaches (i.e. Alternative 1, Alternative 2, or Alternative 3), in which establishments can use in the processing of their RTE products to control *L. monocytogenes*. Under Alternative 1, an establishment must apply a post-lethality treatment and an antimicrobial agent or process

to control subsequent *L. monocytogenes* growth. Under Alternative 2, an establishment must apply either a post-lethality treatment or an antimicrobial agent or process. If an antimicrobial is used on a product manufactured under Alternative 2, then the establishment should maintain sanitation in the post-lethality environment by having a sanitation program. The sanitation program must include food contact surface testing in the post-lethality environment to guarantee the surfaces are free of *L. monocytogenes* or its indicator organism, *Listera spp.* or *Listeria*-like organisms. Under Alternative 3, the establishment does not apply any post-lethality treatment or antimicrobial agent or process; however, it entirely relies on its sanitation program to control *Listeria* (9 CFR Part 430, 2003).

Post-lethality treatments (i.e. steam pasteurization, hot water pasteurization, radiant heating and high pressure processing), have been developed to prevent or eliminate post-processing contamination by *L. monocytogenes.* Post-lethality treatments can be applied as pre-packaging treatment such as radiant heating, or as post-packaging treatments such as hot water or steam pasteurization (9 CFR Part 430, 2003). Antimicrobials are added to foods in order to help control the growth of *L. monocytogenes* in the post-lethality environment. Shelf stable products are formulated with salt, nitrites, and other additives in order to stifle pathogen growth (i.e. water activity < 0.85, pH < 4.5, and moisture-protein ratio). Also, antimicrobials exert continuing bactericidal and bacteriostatic effects in the products, which will enable the product to not support the growth of *L. monocytogenes* during the shelf life of the product. Formulations with added antimicrobials (i.e. lactates and diacetates) are effective growth inhibitors, especially in RTE products such as hotdogs, bologna, cotto salami, and

bratwurst and can be added to the product formulation, to the finished product, or to the packaging material. Many studies have evaluated the effectiveness of dipping or spraying RTE meat and poultry products with antimicrobial solutions including lactic acid, lactate, sodium diacetate and sodium lactate in order to stifle the growth of *Listeria* on finished food product. (Barmpalia et al., 2004; Geornaras et al., 2005; Geornaras et al., 2006; Samelis et al., 2001; Yoon et al., 2009). In one specific study performed by Yoon et al., 2009, the minimum concentrations of lactic acid solutions needed to inhibit *L. monocytogenes* growth on bologna and frankfurters decreased with increasing dipping times, and lower storage temperatures would enhance antimicrobial effects of lactic acid. In addition, longer dipping time was needed to inhibit *L. monocytogenes* growth on frankfurters compared to that for bologna. Models were developed in this study which may be useful in selecting lactic acid concentrations, dipping times, and storage temperatures to control *L. monocytogenes* growth on bologna and frankfurters and in determining the probabilities of growth under the selected conditions, while the modeling procedures presented may be useful for application in various foods, pathogens, and antimicrobial factors (Yoon et al., 2009). Another antimicrobial process that is capable of controlling the growth of *L. monocytogenes* in the post-lethality environment is freezing, which prevents growth by arresting metabolic activities of the bacteria. *L. monocytogenes*; however, is resistant to freezing and once the product is thawed, the metabolic processing may resume depending on the condition (i.e. killed, injured, or unaffected) of the microorganism (9 CFR Part 430, 2003).

Under this rule, establishments will be required to develop effective ways of controlling *Listeria* in RTE products and in the establishment. FSIS will verify the

effectiveness of these control measures (9 CFR Part 430, 2003). Under Alternative 1, the minimum testing is two times per year per production line. Under Alternative 2, the minimum number of testing is four times per year per line. Last is Alternative 3, which has different specifications according to plant size and if products being produced are either non-deli, non-hotdogs or deli, hot-dogs. If the plant operates under Alternative 3 and non-deli/non-hotdog, testing must be performed at least once per month per line. The following specifications are specific for plants which produce products which are deli and hotdogs. For a very small volume plant, testing must be performed at least once per month per line. For small volume plants, testing must be performed at least two times per month per line. For large volume plants, testing must be performed at least four times per month per line (9 CFR Part 430, 2003).

If any of the sites tested results in a positive for *L. monocytogenes* or an indicator organism such as *Listera spp.* then the "hold and test" procedure must be followed, which is incorporated into the sanitation program (9 CFR Part 430, 2003). The "hold and test" procedure that is incorporated into the sanitation program, identifies the conditions under which the establishment will hold product pending test results following an *L. monocytogenes* or an indicator organism positive food contact surface (FCS) test result. If there is a FCS positive for *Listeria* spp., the establishment may conduct an aggressive non-biased sampling method, that the establishment has determined in advance of the suspect product that was in direct contact with this FCS, to determine whether it is *L. monocytogenes* or not. If the sample returns a negative for *L. monocytogenes*, the lot may be released into commerce. If it's determined that the product is *L. monocytogenes*

positive, the establishment may either destroy the product or rework the product using a validated lethality process that will kill *L. monocytogenes* (9 CFR Part 430, 2003).

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CHAPTER II

Molecular ecology of *Listeria monocytogenes* and other *Listeria* species in small and very small ready-to-eat meat-processing plants

Abstract

A longitudinal study was conducted to track *Listeria* contamination patterns in six small or very small ready-to-eat (RTE) meat-processing plants located in three states over one year. A total of 688 environmental sponge samples were collected from non-food contact surfaces during bi-monthly visits to each plant. Overall, *L. monocytogenes* was isolated from 42 (6.1%) environmental samples and its prevalence ranged from 1.7 to 10.8% across different plants. *Listeria* spp., other than *L. monocytogenes,* were isolated from 9.5% of samples overall, with the prevalence ranging from 1.5 to 18.3% across different plants. The prevalence of *L. monocytogenes* correlated well with that of other *Listeria* spp. for some but not all plants. A *L. monocytogenes* isolate representing each positive sample was characterized by molecular serotyping, *Eco*RI ribotyping and pulsed field gel electrophoresis (PFGE) typing. Seven sample sites tested positive for *L. monocytogenes* on more than one occasion and the same ribotype was detected more than once at five of these sites. Partial *sigB* sequencing was used to speciate other *Listeria* spp. isolates and assign an allelic type to each isolate. Other *Listeria* spp. were isolated more than once from 14 sample sites and the same *sigB* allelic type was recovered at least

twice from seven of these sites. One plant was colonized by an atypical hemolytic *L. innocua* strain. Our findings support that small and very small RTE meat-processing plants are characterized by a varied prevalence of *Listeria*, inconsistent correlation between contamination by *L. monocytogenes and* other *Listeria* spp. and a unique *Listeria* molecular ecology.

2.1. Introduction

In a strict sense, the genus *Listeria* is comprised of six species, including *L. monocytogenes, L. innocua, L. welshimeri, L. grayi, L. seeligeri* and *L. ivanovii*, where *L. monocytogenes* represents the only pathogen of public health importance (Rocourt and Buchrieser, 2007). In addition, recent studies proposed that *Listeria marthii* and *Listeria rocourtiae* should be classified as the seventh and eight species in the *Listeria* genus (Graves et al., 2009; Leclercq et al., in press) and have identified atypical hemolytic *L. innocua* strains that carry the *Listeria* pathogenicity island (Johnson et al., 2004). A *L. monocytogenes* infection may lead to a severe invasive disease known as listeriosis, which may manifest as septicemia, meningitis, encephalitis, or abortions/still-births in late-term pregnancies (Senczek et al., 2000). Certain high-risk populations including the elderly, pregnant women, neonates and otherwise immune-compromised individuals are most at risk of sustaining an invasive *L. monocytogenes* infection. The most recent projection indicated that 2,500 cases of listeriosis lead to nearly 500 deaths each year in the United States (Mead et al., 1999). Although *L. monocytogenes* infections are rare compared to the number of illnesses attributed to other foodborne pathogens (such as *Salmonella* and *Campylobacter*), 90% of individuals that sustain an invasive *L. monocytogenes* infection require hospitalization and 20-30% of listeriosis cases are fatal.

Listeriosis accounts for nearly 30% of all deaths attributable to known foodborne pathogens each year in the United States (Mead et al., 1999).

Nearly all listeriosis cases (99%) can be attributed to consumption of foods contaminated by *L. monocytogenes*, where ready-to-eat (RTE) foods are most frequently associated with listeriosis (Farber et al., 1991; Mead et al., 1999). Specifically, the current *L. monocytogenes* risk assessment prepared by the United States Food and Drug Association (USFDA), United States Department of Agriculture Food Safety Inspection Service (USDA:USDA:FSIS), and Centers for Disease Control and Prevention (CDC) implicates RTE deli meats as the overwhelmingly most common food vehicle associated with human listeriosis (estimated to cause nearly 90% of cases). Furthermore, frankfurters that are not reheated before consumption were also grouped into the very high risk category (USDA:FSIS, 2009). Consumption of reheated frankfurters and dry/semi-dry fermented sausages were predicted to represent a moderate risk for listeriosis (USDA:FSIS, 2009). In recent years, multi-state listeriosis epidemics have mostly been attributed to nationally-distributed deli meats and frankfurters produced by large RTE meat-processing plants (Graves et al., 2001; Mead et al., 2006; Olsen et al., 2005). The majority of listeriosis cases, however, are sporadic or occur in small clusters that may not be detected making it difficult to identify the responsible food vehicle and type of facility where the contaminated food was manufactured (Sauders et al., 2003). It stands to reason, however, that small and very small RTE meat plants, which distribute product locally or regionally, may be responsible for a proportion of sporadic cases or small clusters. Taken together these observations highlight the need to provide further information on the molecular ecology and transmission of *Listeria* in RTE meat plants,

where a particular knowledge gap exists for small and very small plants that distribute RTE meat products locally or regionally.

Listeria is ubiquitous in nature and is thus routinely isolated from various environmental sources, including surface water, soil, manure and vegetation (Farber and Peterkin, 1991; Fenlon, 1990). Healthy animals may shed *L. monocytogenes* in their feces and milk for extended periods and raw agricultural commodities are commonly contaminated by this pathogen (Wiedmann et al., 1997). Because *Listeria* is common in raw materials and abundant in nature, the organism will likely be recurrently introduced into the food processing plant environment, through contaminated incoming raw materials. *L. monocytogenes* is easily inactivated by sufficient heat treatment; however, post-processing exposure to the plant environment or other environments (i.e., retail or home) is widely recognized as the predominant source of finished RTE product contamination. Previous studies have demonstrated that the same *L. monocytogenes* strain may persist for extended periods within a given food processing plant environment (Autio et al., 1999; chasseignaux et al., 2001; Destro et al., 1996; Ho et al., 2007; Kabuki et al., 2004; Lappi et al., 2004; Miettinen et al., 1999; Peccio et al., 2003; Shank et al., 1996). The cool and damp to wet conditions in food processing plant environments are conducive for the growth of *Listeria*. *L. monocytogenes* can also establish biofilms in niches in the food processing plant that are difficult to clean and sanitize (Tompkin et al., 1992; FDA/USDA-USDA:FSIS/CDC, 2003).

The public health significance of listeriosis coupled with the foodborne route of *L. monocytogenes* transmission led to the implementation of stringent regulations regarding the presence of this pathogen in finished RTE foods in many countries, including a "zero-

tolerance" policy for the detection of this pathogen in finished RTE foods in the United States (Tenover et al., 1995). The USDA:FSIS requires RTE meat-processing plants to address *L. monocytogenes* control in their hazard analysis critical control point (HACCP) plans, standard operating procedures, and good manufacturing practices plans. Additionally, RTE meat products that are exposed to the food processing plant environment post-processing must be produced under one of three alternatives. Specifically, plants operating under (i) alternative I, must apply microbial inhibitors and incorporate a post-processing lethality treatment, (ii) alternative II, must apply antimicrobial inhibitors or a post-processing lethality treatment, or, (iii) alternative III, must, in addition to a validated cleaning and sanitation program, monitor environmental sanitation through rigorous testing of environmental sites for *Listeria* (Van Stelten and Nightingale, 2008). The USDA:FSIS also conducts routine testing to monitor environmental sanitation. There may be significant implications if an environmental surface that comes into contact with RTE product (food contact surface) after processing in a RTE meat-processing plant environment tests positive for *L. monocytogenes*, and a finished RTE product that tests positive for *L. monocytogenes* will likely elicit a product recall and food safety assessment (Van Stelten and Nightingale, 2008).

Although previous studies investigated the molecular ecology of *Listeria* in seafood and dairy processing plants (Autio et al., 1999; Destro et al., 1996; Ho et al., 2007; Kabuki et al., 2004; Lappi et al., 2004; Miettinen et al., 1991), similar studies in RTE meat-processing plants are more limited due to increased scrutiny regarding the detection of *Listeria* in RTE meat plants. Also, testing for *Listeria* spp. as an indicator for *L. monocytogenes* contamination is routinely practiced but the relationship between

the prevalence of *L. monocytogenes* and other *Listeria* spp. has not been closely evaluated. In addition, knowledge regarding *Listeria* transmission and persistence in small and very small RTE meat plants is limited. The current study was conducted to gain a more comprehensive understanding of *Listeria* contamination patterns in small and very small RTE meat-processing plants. Six small or very small RTE meat-processing plants were enrolled in a longitudinal study where non-food contact surface environmental sponge-swabbing samples were collected bi-monthly over a one-year period. Samples were analyzed to detect *L. monocytogenes* and other *Listeria* spp. and isolates were characterized by molecular subtyping.

2.2. Materials and Methods

Plant Visits and Sample Collection. Six small or very small RTE meat-processing plants were enrolled in this study, where two plants each were located in Colorado, Kansas and Nebraska. At the beginning of the study, a standardized questionnaire was administered to management personnel at each plant to collect basic information regarding plant physical characteristics along with processing and operating characteristics for each plant (Table 2.1). A total of 688 environmental sponge-swabbing samples were collected from non-food contact surfaces bimonthly beginning in February, 2007 through January, 2008 for a total of six sample collections. Environmental sponge samples were collected from approximately 10 to 25 non-food contact surfaces (e.g. walls, doors, drains, floors, sinks, cart wheels and equipment surfaces) mid-shift during each plant visit. All samples were collected with a Whirl-Pak® Speci-Sponge® kit, which includes a sterile sponge, a sterile glove and Whirl-Pak® bag (Nasco, Fort Atkinson,

Wis.). Sponges were first moistened with 10 ml of sterile neutralizing buffer (Solar Biologicals Inc.; Ogdensburg, NY), and then both sides of the moistened sponges were vigorously passed over the sample site surface multiple times. Sponges were placed into a sterile Whirl-Pak® bag, held on ice during transit to the laboratory and were processed within 24 h of collection.

Sample Analysis. Environmental sponge samples were microbiologically analyzed following a modified version of the USDA:FSIS procedure for the use of a *L. monocytogenes* polymerase chain reaction (PCR) screening test (9 CFR Part 430, 2003). Environmental sponge samples were aseptically transferred into a sterile filter Whirl-Pak® stomacher bag (Seward Ltd.; London, UK) and combined with 225 ml of University of Vermont Medium (UVM; Becton Dickinson; Sparks, MD) broth. Samples were then pummeled in a stomacher for 2 min (Stomacher 400; Seward Ltd., West Sussex, UK) and incubated at 30°C ± 2°C for 22 h ± 2 h. UVM enriched-samples (0.1 ml) were streaked onto modified Oxford medium (MOX; Becton Dickinson; Sparks, MD and Oxoid; Hampshire, U.K.) plates and MOX plates were incubated at 35°C ± 2°C for a minimum of 24 h. An aliquot of each UVM enrichment (0.1 ml) was used to inoculate a tube containing 9.9 ml of a secondary morpholinepropanesulfonic acid-buffered *Listeria* enrichment broth (MOPS-BLEB; Becton Dickinson; Sparks, MD). MOPS-BLEB enrichments were incubated at 35°C ± 2°C for 26 h ± 2 h followed by a secondary plating (0.1 ml) onto MOX and incubation of plates as described above.

An aliquot (5 µl) of each MOPS-BLEB enrichment was used to prepare lysates for PCR-based screening to detect *L. monocytogenes* using the BAX® *L. monocytogenes* kit (DuPont Qualicon; Wilmington, DE). Lysate preparation and PCR-based detection of

L. monocytogenes were performed according the manufacturer's instructions (DuPont Qualicon). PCR reactions and melt curve analysis were run on either the ABI PRISM 7000 Sequence Detection System (Applied Biosystems; Foster, CA) or iQ5 Multicolor RT PCR Detection System (Bio-Rad; Hercules, CA). Melt curve results were evaluated for each reaction to ensure detection of a peak corresponding with the internal positive control (IPC; approx. 78°C) and to screen for the presence of a second peak corresponding to detection of a *L. monocytogenes* specific sequence (approx. 84-85°C). Samples that were BAX®-negative (detection of IPC peak only) were reported as negative if the corresponding 24 h primary UVM enrichment plating failed to yield colonies with typical *Listeria* morphology on MOX plates. Cultural analyses was continued for samples that were (i) BAX® -negative but showed colonies with typical *Listeria* morphology resultant from plating the primary 24 h UVM enrichment onto MOX or (ii) BAX®-positive (detection of both the IPC and *L. monocytogenes* sequence specific peaks), –indeterminate or –invalid result. Samples classified as presumptive positive based on the BAX® results and plating of the primary 24 h UVM enrichment were further evaluated by secondary plating onto media that is selective and differential for pathogenic *Listeria*. Specifically, up to four colonies with typical morphology of *Listeria* plated from the 24 h primary UVM enrichment were selected from MOX plates and streaked for isolation onto *Listeria monocytogenes* Plating Medium (LMPM) (R&F Laboratories; Downers Grove, IL.). *L. monocytogenes* and *L. ivanovii* colonies appear with a turquoise-blue color due to hydrolysis of a colorimetric phospholipase substrate, while other *Listeria* spp. form white colonies on LMPM. All presumptive *L. monocytogenes* colonies were confirmed biochemically using an API *Listeria* strip and by *Eco*RI

ribotyping (USDA-USDA-FSIS, 2009). Colonies that appeared white on LMPM and demonstrated typical *Listeria* morphology were further characterized by sequencing a fragment of *sigB* as detailed below. Up to four *L. monocytogenes* colonies and two presumptive *Listeria* spp. colonies from each sample were sub-streaked on brain heart infusion (BHI; Becton Dickson and Company, Sparks, MD) agar plates and cultivated in BHI broth (Becton Dickson; Sparks, MD). *L. monocytogenes* and presumptive *Listeria* spp. isolates were preserved at –80°C in 15% glycerol.

sigB Sequence Typing. Up to two presumptive *Listeria* spp. isolates from each sample presenting colonies typical of *Listeria* spp. other than *L. monocytogenes* were characterized by PCR amplification and sequencing of a fragment of the stress response gene, *sigB*, using previously described reaction conditions and primer sequences (Nightingale et al., 2005). Amplicons were confirmed by agarose gel electrophoresis followed by ethidium bromide staining and visualization under ultraviolet light. PCR products were purified by treatment with ExonucleaseI and Shrimp Alkaline Phosphatase (Fermentas; Glen Burnie, MD) as described previously (Wesley, 1999). DNA sequencing was performed using Big Dye Terminator chemistry and AmpliTaq-FS DNA Polymerase at Colorado State University's Proteomics and Metabolomics Facility (Fort Collins, CO) or at Cornell University's Bioresource Center (Ithaca, NY) as described previously (Nightingale et al., 2005). DNA sequences were assembled, proofread, and aligned using Seqman and Megalign (Lasergene; Madison, WI). Sequence data for all isolates are available through the PathogenTracker database (www.pathogentracker.net). BLAST searches were performed for each *sigB* sequence to determine the species of each *Listeria* spp. isolate. An alignment of all *sigB* sequences was imported into DnaSP

(Rozas et al., 2003) and a *sigB* allelic type, defined as a unique combination of polymorphisms within *sigB*, was assigned to each isolate belonging to *Listeria* other than *L. monocytogenes*. Sequencing *sigB* and allelic typing was previously shown to be concordant with API *Listeria* test strips for *Listeria* identification purposes (Sauders et al., 2006).

Molecular Serotyping. A single *L. monocytogenes* isolate representing each *L. monocytogenes* positive sample (n=42) from the six RTE meat plants enrolled in this study was selected for further molecular characterization. *L. monocytogenes* isolates were characterized by a multiplex PCR assay to group isolates into molecular serogroups (Doumith et al., 2004), where each molecular serogroup contains one of the four major serotypes associated with human disease (1/2a, 1/2b, 1/2c and 4b). This assay detects the presence of four serotype-specific marker genes (*lmo*0737, *lmo*1118, ORF2819, ORF2110) and amplifies *prs*, which is found in all *Listeria* spp., as an internal control. *L. monocytogenes* isolates that were characterized by conventional serotyping as serotypes 1/2a, 1/2b, 1/2c, and 4b were included in each reaction as controls. Isolates were grouped into molecular serogroups based on the presence or absence of each target included in the multiplex PCR assay as detailed previously (Doumith et al., 2004).

Ribotyping. Automated *Eco*RI ribotyping was performed to confirm and characterize one isolate from each presumptive *L. monocytogenes*-positive sample essentially as described by Bruce et al. (1996). Ribotyping was performed at Cornell University's Laboratory of Molecular Subtyping (Ithaca, NY) using the automated Riboprinter® (DuPont Qualicon). Briefly, total bacterial DNA was lysed and loaded into the Riboprinter®. Bacterial DNA was cleaved by *Eco*RI and resultant DNA fragments were

separated by size. DNA fragments were captured and immobilized on a nylon membrane, and a chemically labeled rRNA operon probe was used to identify fragments containing rRNA genes. Fragment pattern data were normalized using custom software and DuPont ID numbers (e.g., DUP-1052) were given to each isolate. If patterns differed by one single weak band from an already assigned DuPont ID, then each pattern was designated by addition of an alphabetized letter (e.g., DUP-1052A and DUP-1052B). Ribotype patterns for each isolate characterized in this study are available through PathogenTracker (www.pathogentracker.net).

Pulsed Field Gel Electrophoresis (PFGE) typing. One isolate from each *L. monocytogenes*-positive sample was characterized by PFGE typing. PFGE typing was performed using the standardized Centers for Disease Control and Prevention (CDC) PulseNet protocol. Briefly, *L. monocytogenes* isolates were grown on BHI agar plates at 37°C for 18 hours. Bacterial cultures at optimized optical densities were imbedded in 1% SeaKem Gold® agarose (Cambrex Bio Science, Rockland, ME), lysed, washed, and digested separately with *Asc*I for 5.5 h at 37°C and *Apa*I for 7 h at 30°C. Restricted agarose plugs were then placed into 1.5% agarose gels and electrophoresed on a CHEF Mapper XA (BioRad Laboratories) for 21 h with switch times of 4.0 s to 40.0 s. *Xba*I digested *Salmonella* ser. Braenderup (H9812) DNA was used as a reference size standard (Hunter et al., 2005). Agarose gels were stained in ethidium bromide and resultant images were captured with a Gel Doc™ XR (BioRad Laboratories; Hercules, CA) or FOTO/Analyst Investigator System (FOTODYNE; Inc., Hartland, WI). PFGE patterns were analyzed and compared using the Applied Maths BioNumerics (Applied Maths; Saint-Matins-Latem, Belgium) software package. Similarity clustering analyses were

performed with BioNumerics software using the unweighted pair group matching algorithm and the Dice correlation coefficient as described previously (Hunter et al., 2005).

2.3. Results

Plant 1. Based on six bi-monthly sample collections throughout one year, 10.8% of 157 environmental samples from plant 1 tested positive for *L. monocytogenes*, while 15.9% of samples contained *Listeria* spp. other than *L. monocytogenes* (Table 2.2). While the majority of *L. monocytogenes* isolates from plant 1 were classified into the molecular serogroup containing serotypes 1/2b, 3b and 7, a few isolates fell into the other molecular serogroups, including the serogroups of 1/2a and 3a and 1/2c and 3c (Table 2.3). Both *Eco*RI ribotyping and PFGE typing were used to characterize *L. monocyogenes* as each subtyping approach has unique benefits for tracking contamination patterns with the food processing plant environment. Specifically, ribotyping is particularly useful for tracking contamination patterns of strains that are genetically related at the core level as changes in rRNA genes accumulate slowly over time, while PFGE can be employed to monitor divergence of strains colonizing the environment of a specific food processing plant as PFGE probes genetic changes that occur more rapidly in a strain (e.g., mutations in auxiliary genes as well as the gain/loss of plasmids) (Carlson et al., 2009). The 17 *L. monocytogenes* isolates from plant 1 characterized by *Eco*RI ribotyping were classified into five unique ribotypes, with the majority of isolates belonging to ribotype DUP-1052A (Table 4). Two isolates belonging to ribotype DUP-18602 and a single isolate each belonging to ribotypes DUP-1042B and DUP-18616 were detected in the raw/in-process areas of this plant, and one isolate, representing DUP-1048A, was found on the

floor in the finished product packaging area (Table 2.4). *L. monocytogenes* isolates belonging to ribotype DUP-1052A persisted in plant 1 over the duration of this sampling period and this strain was detected in the ice machine and stuffing area floor during two different sample collections. For the most part, DUP-1052A was contained in the raw/in-process area, suggesting that this strain may have been continuously re-introduced into this plant from raw in-coming materials. However, *L. monocytogenes* belonging to ribotype DUP-1052A was isolated from the finished product handling area and finished product storage cooler during one sample collection (Table 2.4).

Characterization of the 17 *L. monocytogenes* isolates from plant 1 by PFGE typing revealed 12 different combined *Apa*I/*Asc*I pulsotypes (Table 2.3). Since *Apa*I restriction patterns were more discriminatory than *Asc*I patterns, dendrograms of *Apa*I digested fragment patterns were used to visualize clustering of isolates within each plant and to assess the relatedness of other strains within the same plant. In plant 1, 8 isolates belonged to a predominant *Apa*I PFGE type (*Apa*I pattern type A; Fig. 2.1); this predominant *Apa*I PFGE pattern was used as the reference type in order to classify other isolates as closely related (three or fewer band difference between two patterns), possibly related (four to six band difference) or distantly related (more than seven band difference) to the predominant strain according to the criteria specified by Tenover et al. (1995). Based on this analysis, three isolates were closely related to the predominant *Apa*I pattern and six isolates were not related to the most common *Apa*I pattern observed among isolates from plant 1 (Fig. 2.1).

BLAST searches for *sigB* sequences were performed to speciate isolates belonging to *Listeria* spp. other than *L. monocytogenes*. BLAST search results showed

that *L. innocua* and *L. welshimeri* were present in the environment of plant 1 (Table 2.4). Unique combinations of *sigB* polymorphisms were used to assign *sigB* allelic types to *Listeria* spp. isolates. *Listeria* spp. isolates from plant 1 grouped into six different *sigB* allelic types. *L. innocua* isolates belonging to *sigB* allelic type 56 (AT56; Table 2.4) were predominant in the environment of plant 1 (n=16), followed by *L. welshimeri* AT19 isolates (n=5). The same allelic type (*L. innocua* AT56) was isolated from the drain in the stuffing area on three separate occasions. In addition, the same *Listeria* spp. allelic type was isolated twice from four other sites in the raw area, including chopper controls (*L. welshimeri* AT19), stuffing area floor (*L. innocua* AT56), raw area smoketruck wheels (*L. innocua* AT56), and the door separating the smokehouse room from the finished product packaging room (*L. welshimeri* AT19) (Table 2.4).

Plant 2. Results from environmental testing showed that *L. monocytogenes* was isolated from 6.9% of the 160 samples tested, and *Listeria* spp. other than *L. monocytogenes* were isolated from 5.0% of samples (Table 2.2). Nine of the 11 *L. monocytogenes* isolates from plant 2 belonged to the molecular serogroup containing serotypes 1/2b, 3b, and 7, while the remaining two isolates were classified into a molecular serogroup that includes serotypes 1/2a and 3a (Table 2.3). Characterization of *L. monocytogenes* isolates from plant 2 by *Eco*RI ribotyping revealed three ribotypes, where eight isolates belonged to a predominant ribotype (DUP-1052A). Two isolates were characterized as ribotype DUP-18627 and one isolate belonged to ribotype DUP-1042B (Table 2.5). *L. monocytogenes* isolates belonging to ribotype DUP-1052A were isolated during three of the six sampling collections. During the sample collection on 5/17/07, ribotype DUP-1052A was isolated from five different raw/in-process sites located across both buildings and was transmitted

to the fabrication area hand sink in the packaging area (Table 2.5). Ribotype DUP-1052A was isolated on multiple occasions from two drains in the raw/in-process area of plant 2 (i.e., drains in the grinding area and preparation area) and the drip area drain tested positive for *L. monocytogenes* on two different occasions, suggesting that *L. monocytogenes* persisted in the drains of this plant. PFGE typing using restriction by *Apa*I and *Asc*I grouped the 11 *L. monocytogenes* isolates from plant 2 into four different combined pulsotypes (Tables 2.3 & 2.5). The majority of the *L. monocytogenes* isolates grouped within the same *Apa*I pulsotype (*Apa*I pulsotype H) and the remaining *Apa*I pulsotypes observed with isolates from plant 2 were not related to the predominant pulsotype (Tables 2.3 & 2.5).

Listeria spp. other than *L. monocytogenes* were speciated and assigned allelic types using *sigB* sequence data. The predominant species of *Listeria* detected in plant 2 was *L. welshimeri* and only two *L. innocua* isolates were detected (Table 2.5). Four *sigB* allelic types were found overall with allelic type 27 (*L. welshimeri* AT27) representing the most common *sigB* allelic type. AT27 was isolated in both the raw/in-process area and in-process/finished areas during the same sample collection. All other *sigB* allelic types isolated from plant 2 were only isolated from the raw/in-process areas. *Listeria* spp. other than *L. monocytogenes* did not appear to persist in the environment of plant 2 as each unique *sigB* allelic type was only isolated during a single sample collection.

Plant 3. Over the one-year sampling period, only two out of the 70 collected samples tested positive for *L. monocytogenes* and seven samples tested positive for *Listeria* spp. other than *L. monocytogenes* (Table 2.2). Molecular serotyping showed that *L. monocytogenes* isolates could be assigned into a molecular serogroup containing the most

common human disease associated *L. monocytogenes* serotypes (Table 2.3). The two *L. monocytogenes* isolates belonged to ribotype DUP-1061A (Table 2.6), which group within the rare *L. monocytogenes* lineage III (Wiedmann et al., 1997). Characterization of *L. monocytogenes* isolates by PFGE typing showed that both isolates belonged to two closely related *Apa*I pulsotypes (Fig. 2.1). Analyses of *sigB* sequences identified *Listeria* spp. isolates other than *L. monocytogenes* from plant 3 as hemolytic *L. innocua* (Sauders et al., 2003). The same hemolytic *L. innocua* strain AT87 was isolated from multiple environmental sites from raw/in-process/finished and raw/finished areas in plant 3 during the last two sample collections (Table 2.6).

Plant 4. *L. monocytogenes* was detected in 7.6% of 66 environmental samples, while *Listeria* spp. other than *L. monocytogenes* were detected in 1.5% of samples. All samples that tested positive for *Listeria* were from the final sample collection. Molecular characterization of the five *L. monocytogenes* isolates from plant 4 showed that all isolates belonged to the same molecular serogroup (1/2b, 3b, 7), ribotype (DUP-1025B) and combined *Apa*I/*Asc*I pulsotype (combined pulsotype 18; Tables 2.3 & 2.7). Only one other *Listeria* spp. isolate was detected from a sponge sample of the cooler light switch in the raw/in-process/finished area and *sigB* allelic typing classified this isolate as *L. welshimeri* AT89 (Table 2.7).

Plant 5. *L. monocytogenes* was detected in 4.4% of 115 samples collected over the one-year period and *Listeria* spp. other than *L. monocytogenes* were detected in 1.7% of the environmental samples (Table 2.2). All five *L. monocytogenes* isolates belonged to the molecular serogroup that contains serotypes 1/2a and 3a (Table 2.3). *L. monocytogenes* isolates from plant 5 represented four different ribotypes (Table 2.8) and four different

combined *Apa*I/*Asc*I pulsotypes (Table 2.3). Four of the five *L. monocytogenes* isolates were detected during the fourth sample collection on 9/19/07, where the same ribotype (DUP-1053A) was isolated from the packside drain and stress mats located adjacent to the packside drain (Table 2.8). *L. welshimeri* AT 69 was isolated from the individual quick freeze belt and pallet jack wheels once each during the first two sample collections (Table 2.8).

Plant 6. Interestingly, plant 6 showed the lowest prevalence of *L. monocytogenes* (1.7%) but the highest prevalence of *Listeria* spp. other than *L. monocytogenes* (18.3%; Table 2.2) as compared to the other five plants in this study. Both *L. monocytogenes* isolates from this plant belonged to the same molecular serogroup (i.e., serotypes 1/2c and 3c), ribotype (DUP-1039C), and combined *Apa*I/*Asc*I pulsotype (combined pulsotype 23; Table 3). The two *L. monocytogenes* positive samples were from the trench floor drain and wheels of the slicer cart in the finished product packaging area (Table 2.9). Analyses of *sigB* sequences showed that five different *L. innocua sigB* allelic types and one *L. welshimeri sigB* allelic type were present in the environment of this plant. *L. innocua* AT 70 was the most common *sigB* allelic type (n=11) followed by *L. innocua* AT31 (n=5), *L. innocua* AT 53 (n=3). All other *sigB* allelic types were unique to a single isolate (Table 2.9). One specific site (i.e., smokehouse room trough floor drain) was colonized by AT70 over multiple sample collections in the raw/in-process area. Also, *Listeria* spp. other than *L. monocytogenes* were isolated from four specific sites (i.e., floor at smokehouse door, trench floor drain by south smokehouse, squeegee used for floors in the entire plant, and cooked meat cooler floor drain) more than once but *Listeria* isolates from each site belonged to different *sigB* allelic types.

2.4. Discussion

A one-year longitudinal study was conducted to probe the molecular ecology of *Listeria* in six small or very small meat-processing plants. Results of this study showed that (i) the prevalence of *L. monocytogenes* and *Listeria* spp. other than *L. monocytogenes* varied across the six small and very small RTE meat plants enrolled in the study, (ii) the presence of *L. monocytogenes* and other *Listeria* spp. in the plant environment correlated well for some but not all plants and (iii) consistent with large RTE meat-processing plants, each small or very plant appeared to be colonized by a single or few predominant *Listeria* strains. Results from this study highlight the critical need for stringent employee practices along with cleaning and sanitation procedures to eliminate harborage sites and disrupt transmission of *L. monocytogenes* from raw/in-process areas to finished product areas.

The prevalence of *L. monocytogenes* and *Listeria* spp. other than *L. monocytogenes* varies across plants. The one-year prevalence of *L. monocytogenes* ranged from 1.7% to 10.8% and the prevalence of *Listeria* spp. other than *L. monocytogenes* ranged from 1.5% to 18.3 % across the six plants enrolled in the current study. Overall, the majority of samples testing positive for *L. monocytogenes* were obtained from the environments of plants 1 and 2, while plants 1 and 6 showed the highest number of samples testing positive for *Listeria* spp. other than *L. monocytogenes*. These results are consistent with a previous study that described considerable variation in prevalence of *Listeria* in the environment of 13 small dried sausage processing plants before and during operations (Tompkin, 2002). Data from the questionnaires administered to the plants enrolled in this study showed that plants 1, 2, and 5 produced a notably larger volume of RTE meat

products as compared to the other plants in this study. Plants 1 through 5 operated at least five days each week year-round, while plant 6 only processed a few days a week on an as needed basis (Table 2.1). Interestingly, a previous study by Lappi and co-workers (Lappi et al., 2004) described an anecdotal correlation between plant operation patterns, moisture levels and detection of *Listeria* in the plant environment. Specifically, *L. monocytogenes* was not isolated from the environment of one smoked seafood plant that primarily produced retort processed products packaged in hermetically sealed containers and only processed refrigerated products one or two days per week. The authors rationalized that allowing the plant to dry out for several days at a time in between the wet processing cycles for refrigerated product may have deterred *L. monocytogenes* persistence (Lappi et al., 2004). In future studies, it may prove valuable to (i) probe associations between production volume and environmental *Listeria* contamination levels and (ii) collect data on plant moisture levels and sample site moisture levels to more precisely quantify the association between moisture and *Listeria* persistence. Our results indicate that the prevalence of *Listeria* varies considerably across small and very small RTE meat-processing plants and suggest that increased production volume and continuous operation may be associated with increased levels of *L. monocytogenes* contamination.

The presence of *L. monocytogenes* and *Listeria* spp. other than *L. monocytogenes* correlated well for some but not all plants. There have been considerable discussions regarding the issue of testing for *Listeria* spp. as an indicator of *L. monocytogenes* contamination (Tompkin, 2002). Tompkin (1992) advocated that plants opting to test for *Listeria* spp. without further microbiological analyses to determine if *Listeria* spp.

positive samples contain *L. monocytogenes* should treat all *Listeria* spp. positive as if they were confirmed to be *L. monocytogenes* positive in order to be proactive about a potential sanitation failure and resultant problems. Based on the results from our study, this approach would be practical for plants with a very low number of *Listeria* positive samples, particularly if most *Listeria* positive samples were confirmed as *L. monocytogenes*. More specifically, plants 4 and 5 had a small number of samples that were positive for *Listeria* and most of these samples were confirmed to be *L. monocytogenes*; testing for *Listeria* spp. would be a good indicator for *L. monocytogenes* for these two plants (Table 2.2). On the other hand, two plants (plants 3 and 6) had notably higher numbers of samples that were positive for other *Listeria* spp. and a small number of samples testing positive for *L. monocytogenes*. Lastly, plants 1 and 2 were characterized by an overall elevated number of environmental sites that tested positive for both *L. monocytogenes* and other *Listeria* spp. as compared to the other plants enrolled in this study. In addition, only four environmental samples contained both *L. monocytogenes* and another *Listeria* spp. over the duration of the study (Tables 2.4 – 2.9). Another previous study by Tompkin et al. (1992) also revealed an inconsistent relationship between the presence of *L. monocytogenes* and other *Listeria* spp. in the environment of 12 large RTE meat-processing plants. While the majority of *Listeria* positive samples were confirmed as *L. monocytogenes* for three plants, < 50% of *Listeria* positive samples contained *L. monocytogenes* for the remaining nine large RTE meat plants enrolled in the study, where < 2% of 318 *Listeria* positive samples were confirmed as *L. monocytogenes* for one plant (Tompkin et al., 1992). An individual RTE meat-processing plant, regardless of size, appears to be characterized by a unique *Listeria*

ecology. Specifically, some plants appear to be colonized by both *L. monocytogenes* and other *Listeria* spp., while other plants seem to be predominantly colonized by either *L. monocytogenes* or other *Listeria* spp. Collectively, results from this study and previous studies, support the implications for finding a sample that tests positive for *Listeria* spp. may depend on the ecology of that plant, where the presence of a non-pathogenic *Listeria* spp. does not necessarily correlate with the presence of *L. monocytogenes*. Because small and very small plants have restricted resources, plants with a history of having a significant number of environmental sites testing positive for *Listeria* spp. should determine whether or not those samples contain *L. monocytogenes* to prioritize efforts to directly control *L. monocytogenes* in the plant environment. However, detection of any *Listeria* spp. may indicate a sanitation failure and should be addressed to prevent future problems.

Small and very small RTE meat plants appear to be characterized by a single or few predominant *Listeria* strains. Previous studies demonstrated the utility of combined routine environmental monitoring for *Listeria* and molecular subtyping of isolates to identify harborage sites and monitor contamination patterns within a given RTE food processing plant (e.g., Autio et al., 1999; chasseignaux et al., 2001; Destro et al., 1996; Ho et al., 2007; Kabuki et al., 2004; Lappi et al., 2004; Miettinen et al., 1999; Peccio et al., 2003; Shank et al., 1996). Plants 1 and 2 were predominantly colonized by *L. monocytogenes* isolates belonging to ribotype DUP-1052A and this strain persisted in the raw/in-process areas of both plants throughout the study and was also transmitted to the finished product packaging areas in both plants during the second sample collection at each plant (Tables 2.4 & 2.5). During the second sample collection at plant 1, *L.*

monocytogenes belonging to ribotype DUP-1052A may have been transmitted from the smokehouse rooms to the finished product area on the smokehouse cart-wheels, where it then was isolated from three more sites in the finished product area (Table 2.4). In plant 2, ribotype DUP-1052A isolates were found in raw/in-process areas in the main building, auxiliary building where RTE sausage is smoked and dried and area where processed meat products are packaged. Throughout the study, testing results were communicated to each plant in a real-time manner and plants 1 and 2 implemented interventions after learning that the same *L. monocytogenes* strain was transmitted from the raw/in process areas to areas where finished RTE products were packaged in those plants (Tables 2.4 & 2.5). Specifically, plant 1 installed a door foamer between the smokehouse room and the finished product packaging room and plant 2 implemented intensive cleaning and sanitation to interrupt transmission of *L. monocytogenes* from raw/in-process areas to finished product areas. Interestingly, *L. monocytogenes* was not isolated from the environment of the finished product handling areas in either plant 1 or 2 after these interventions were implemented. Plant 3 was colonized by an atypical hemolytic *L. innocua* strain (*sigB* allelic type 87), which was isolated from the same drain in the finished product cooler during the last two consecutive sample collections (Table 2.6). *L. monocytogenes* was only detected in plant 4 during the final sample collection and all isolates belonged to the same strain (ribotype DUP-1025B). Plant 5 appeared to be transiently contaminated by *L. monocytogenes*, as each strain was only observed during a single sample collection, but the same *L. welshimeri sigB* allelic type was detected over consecutive sample collections. A few *L. innocua* strains (AT70, AT31 and AT53) appeared to have become persistently established in the environment of plant 6. In

particular, one strain (*L. innocua* AT70) was isolated from the environment of plant 6 over five consecutive sample collections and this strain was also transmitted from the raw and in-process areas to finished product handling areas over four consecutive sample collections (Table 2.9). The same *L. monocytogenes* strain (ribotype DUP-1039C) was isolated from two sites in the finished product area, including the wheels on the slicer cart, during one sample collection.

Across all six plants *Listeria* contamination was primarily associated with raw/in-process areas; however, certain subtypes were found in these areas on more than one occasion. Some sites within environment of all plants (e.g., floors and drains) appeared to particularly problematic with respect to repeated isolation of the same *Listeria* subtype. Plants should target their cleaning and sanitation efforts towards these sites in order to deter persistence and reduce the risk of transmission to finished product areas. Previous studies have described an association between persistence of *L. monocytogenes* in non-food contact surfaces of the plant and finished RTE product contamination (e.g., *16*), highlighting the utility of a routine environmental monitoring program that includes microbiological testing to detect *L. monocytogenes* and molecular subtyping to identify harborage sites. Consistent with previous studies, our findings support that each plant appears to be characterized by a unique *Listeria* ecology, where even small and very small plants appear to become persistently colonized by a single or few predominant *Listeria* strains.

2.5. Conclusions.

The prevalence of *L. monocytogenes* and other *Listeria* spp. varied considerably across the small or very small RTE meat-processing plants enrolled in this study and the

environmental presence of *L. monocytogenes* and other *Listeria* only correlated for some plants. As a result, small and very small RTE meat-processing plants, which have fewer resources than large plants, should consider further analyzing samples that test positive for *Listeria* spp. to confirm *L. monocytogenes* as testing for *Listeria* spp. as an indicator for *L. monocytogenes* would not be beneficial or practical for some plants. However, detection of *Listeria* spp. in the plant environment, and particularly isolation of the same molecular subtype from the same environmental site on more than one occasion, should be recognized as a cleaning and sanitation failure and a plant should take proactive measures to eliminate harborage sites. Molecular subtyping showed that a single or few predominant *L. monocytogenes* or other *Listeria* spp. strains can persist in environment of small and very small RTE meat plants, highlighting the need for stringent cleaning and sanitation efforts to prevent cross-contamination of RTE meat products exposed to the environment following the lethality step. Results from this study support the usefulness of a combined testing and molecular subtyping approach to conduct routine environmental monitoring of *Listeria* contamination in small and very small RTE meat-processing plants and real-time communication of results to interrupt transmission of *L. monocytogenes* from raw/in-process areas to RTE meat packaging areas.

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TABLE 2.1. Description of small and very small ready-to-eat meat plant demographics

Plant	No. Employees (type)[a]	Square Footage	Pounds produce / year	Product produced	Slaughter included	Location	Age	Alternative[b]
Plant 1	12(FT)	7,800	500,000 lbs	RTE meat	No	Urban	26 years	3
Plant 2	15(FT)	10,500	600,000 lbs	Fresh meat, RTE meat	Yes	Suburban	13-68 years	2
Plant 3	7(FT) 4(PT)	20,000	4,500 lbs	Fresh meat, RTE meat	Yes	Suburban	42 years	3
Plant 4	5(FT) 1(PT)	3,150	30,000 lbs	Fresh meat, RTE meat	Yes	Rural	48 years	3
Plant 5	29(FT) 46(PT)	65,000	1,089,900 lbs	RTE meat	No	Rural	17 years	2
Plant 6	18(FT) 2(PT) 2(SE)	14,000	100,000 lbs	Fresh meat, RTE meat	Yes	Rural	45 years	3

[a] FT indicates full-time; PT indicates part-time; SE indicates seasonal employees.

[b] Indicates which of the three alternatives that each plant manufactured RTE meat products under. Specifically, plants operating under (i) alternative I, must apply microbial inhibitors and incorporate a post-processing lethality treatment, (ii) alternative II, must apply antimicrobial inhibitors or a post-processing lethality treatment, or, (iii) alternative III, must, in addition to a validated cleaning and sanitation program, monitor environmental sanitation through rigorous testing of environmental sites for *Listeria*

TABLE 2.2. Summary of *Listeria* prevalence in small and very small ready-to-eat meat processing plants

		No. *Listeria* positive samples	
Ready-to-eat meat processing plant	No. samples collected	No. samples positive for *L. monocytogenes* (% positive)	No. samples positive for other *Listeria* spp. (% positive)[a]
Plant 1	157	17 (10.8)	25 (15.9)
Plant 2	160	11 (6.9)	8 (5.0)
Plant 3	70	2 (2.9)	7 (10.0)
Plant 4	66	5 (7.6)	1 (1.5)
Plant 5	115	5 (4.5)	2 (1.7)
Plant 6	120	2 (1.7)	22 (18.3)
All plants	688	42 (6.1)	65 (9.5)

[a] Indicates number of samples that tested positive for *Listeria* spp. other than *L. monocytogenes*

TABLE 2.3. Summary of molecular characteristics of a single *Listeria monocytogenes* isolate selected to represent each *L. monocytogenes* positive sample.

Isolate	ApaI pulsotype	AscI pulsotype	Combined ApaI/AscI pulsotype	EcoRI ribotype[a]	Molecular serogroup[b]	Plant	Sample collection
CSUFSL W1-159	B	K	1	DUP-1052A	1/2b, 3b or 7	Plant 1	3
CSUFSL W1-211	F	O	2	DUP-18616	1/2a or 3a	Plant 1	4
CSUFSL W1-215	A	H	3	DUP-1052A	1/2b, 3b or 7	Plant 1	4
CSUFSL W1-224	A	H	3	DUP-1052A	1/2b, 3b or 7	Plant 1	4
CSUFSL W1-231	A	H	3	DUP-1052A	1/2b, 3b or 7	Plant 1	4
CSUFSL W1-273	A	H	3	DUP-1052A	1/2b, 3b or 7	Plant 1	5
CSUFSL W1-345	B	H	4	DUP-1052A	1/2b, 3b or 7	Plant 1	6
CSUFSL W1-111	A	I	5	DUP-1052A	1/2b, 3b or 7	Plant 1	2
CSUFSL W1-113	A	I	5	DUP-1052A	1/2b, 3b or 7	Plant 1	2
CSUFSL W1-163	A	K	6	DUP-1052A	1/2b, 3b or 7	Plant 1	3
CSUFSL W1-121	A	L	7	DUP-1052A	1/2b, 3b or 7	Plant 1	2
CSUFSL W1-129	B	L	8	DUP-1052A	1/2b, 3b or 7	Plant 1	2
CSUFSL W1-353	C	P	9	DUP-1052A	1/2b, 3b or 7	Plant 1	6
CSUFSL W1-057	D	M	10	DUP-1042B	1/2b, 3b or 7	Plant 1	1
CSUFSL W1-049	E	N	11	DUP-18602	1/2a or 3a	Plant 1	1
CSUFSL W1-137	E	N	11	DUP-18602	1/2a or 3a	Plant 1	2
CSUFSL W1-041	G	Q	12	DUP-1048A	1/2c or 3c	Plant 1	1

CSUFSL W1-061	A	L	7	DUP-1052A	1/2b, 3b or 7	Plant 2	1
CSUFSL W1-075	H	R	13	DUP-1052A	1/2b, 3b or 7	Plant 2	2
CSUFSL W1-083	H	R	13	DUP-1052A	1/2b, 3b or 7	Plant 2	2
CSUFSL W1-087	H	R	13	DUP-1052A	1/2b, 3b or 7	Plant 2	2
CSUFSL W1-091	H	R	13	DUP-1052A	1/2b, 3b or 7	Plant 2	2
CSUFSL W1-099	H	R	13	DUP-1052A	1/2b, 3b or 7	Plant 2	2
CSUFSL W1-107	H	R	13	DUP-1052A	1/2b, 3b or 7	Plant 2	2
CSUFSL W1-167	H	R	13	DUP-1052A	1/2b, 3b or 7	Plant 2	3
CSUFSL W1-341	I	F	14	DUP-1042B	1/2b, 3b or 7	Plant 2	6
CSUFSL W1-325	J	A	15	DUP-18627	1/2a or 3a	Plant 2	5
CSUFSL W1-333	J	A	15	DUP-18627	1/2a or 3a	Plant 2	5
CUFSL R8-021	K	G	16	DUP-1061A	Untypeable	Plant 3	6
CUFSL R8-052	L	G	17	DUP-1061A	Untypeable	Plant 3	6
CUFSL R8-035	M	E	18	DUP-1025B	1/2b, 3b or 7	Plant 4	6
CUFSL R8-037	M	E	18	DUP-1025B	1/2b, 3b or 7	Plant 4	6
CUFSL R8-041	M	E	18	DUP-1025B	1/2b, 3b or 7	Plant 4	6
CUFSL R8-045	M	E	18	DUP-1025B	1/2b, 3b or 7	Plant 4	6
CUFSL R8-049	M	E	18	DUP-1025B	1/2b, 3b or 7	Plant 4	6
CUFSL R6-651	N	C	19	DUP-1053A	1/2a or 3a	Plant 5	4
CUFSL R6-653	N	D	20	DUP-1053A	1/2a or 3a	Plant 5	4
CUFSL R6-643	O	A	21	DUP-1062A	1/2a or 3a	Plant 5	4

CUFSL R6-646	O	A	21	DUP-1062D	1/2a or 3a	Plant 5	4
CUFSL R6-555	P	B	22	DUP-1056A	1/2a or 3a	Plant 5	3
CUFSL R6-484	Q	J	23	DUP-1039C	1/2c or 3c	Plant 6	2
CUFSL R6-489	Q	J	23	DUP-1039C	1/2c or 3c	Plant 6	2

[a] When an assigned DuPont ID included more than one distinct ribotype pattern (e.g., patterns differing by a single weak band), each pattern was designated with an additional alphabetized letter (e.g., DUP-1039A and DUP-1039B)
[b] Isolates were classified into a molecular serogroup using a multiplex PCR assay previously described by Doumith et al. (2004).

TABLE 2.4. *Listeria monocytogenes* and *Listeria spp.* contamination profiles for Plant 1 over a one-year period with bi-monthly sample collections.

Plant 1						
Sample Location	Dates Sampled					
	3/8/07	5/30/07	8/8/07	9/19/07	11/14/07	1/15/08
Raw/In-Process Area						
Pallet Jack Handle	.	.	.	.	.	.
Raw Area to Packaging Area Door	.	.	.	.	LI AT56	.
Raw Area Ice Machine	.	.	LM DUP-1052A	.	LI AT56	LM DUP-1052A
Time Clock	NS	NS	NS	.	NS	NS
Grind/Chop Area Floor	.	.	.	LM DUP-1052A	LI AT56	.
Raw Area Hand Sink	.	.	.	.	LI AT56	.
Raw Area Apron	.	.	LI AT56	.	LI AT19	.
Raw Area Chopper Controls	.	.	LI AT19	.	LI AT19	LM DUP-1052A
Chop/Grind Area Drain	.	LW AT27	LI AT56	.	LM DUP-1052A	.
Stress Mat in Brine Area	NS	NS	.	NS	NS	NS
Stuff Area Floor	LM DUP-1042B	LW AT69	LM DUP-1052A	LM DUP-1052A	LI AT56	LI AT56
	.	.	LI AT56	.	.	.
Raw Area Smktruck Wheels	.	.	LI AT56	.	LI AT56	.
Stuff Area Drain	LM DUP-18602	.	LI AT56	LM DUP-1052A	LI AT56	LI AT56
Stuff Area to Smoke Door	.	LI AT109	LI AT19	.	LI AT19	.

Door to Packaging Area	.	.	.	.	.	.
Smokehouse to Packaging Door	.	.	.	LM DUP-18616	.	.
Shower Area Drain	.	.	.	.	.	.
Smokehouse Drain	.	LI AT110	.	.	LI AT56	.
Smokehouse #1 Door Handle	.	LM DUP-18602	.	.	.	.
	.	LI AT56	.	.	.	.
Smokehouse # 2 Handle	NS	NS	.	NS	NS	NS
Smokehouse Control Panel	NS	NS	NS	.	NS	NS
Finished Product Area						
Packaging Area Drain #1	.	.	.	.	.	.
Packaging Area Drain #2	NS	.	NS	NS	NS	NS
Packaging to Cooler Door	.	.	.	.	.	.
Smoketruck Wheels	.	LM DUP-1052A	.	.	.	.
Packaging to Dock Door	.	LM DUP-1052A	.	.	.	.
Vacuum Packager Lid and Controls	.	.	.	.	.	.
Packaging Area Hand Sink	.	LM DUP-1052A	.	.	.	.
Packaging Area Floor	LM DUP-1048A	.	.	.	.	.
Cooler Box Cart Wheels	NS	NS	NS	.	NS	NS
Cooler Floor	.	LM DUP-1052A	.	.	.	.
Cooler to Dock Door	NS	NS	NS	.	NS	NS

[a] NS indicates a site that was not sampled during a given sample collection and a "-" indicates that a sample did not test positive for *Listeria* during a given sample collection. *Listeria* positive samples are described by two capital letters to indicate the species that was isolated from the sample; where LM indicates *L. monocytogenes*, LI indicates *Listeria innocu*a, HLI indicates hemolytic *L. innocua*, and LW indicates *L. welshimeri*. *L. monocytogenes* molecular subtype is denoted (e.g., *Eco*RI ribotype DUP-1052A) and the *Listeria* spp. *sigB* allelic type (e.g., AT23) is denoted also denoted following species designation.

TABLE 2.5 *Listeria monocytogenes* and *Listeria spp.* contamination profiles for Plant 2 over a one-year period with bi-monthly sample collections.

Plant 2						
Sample Location	Dates Sampled					
	3/20/07	5/17/07	8/9/07	9/17/07	11/14/07	1/15/08
Raw/In-Process Areas-Main Facility Building						
Shipping Dock Table	.	.	.	.	.	.
Drip Area Floor	.	LM DUP-1052A	.	.	LM DUP-18627	.
Drip Area Drain	.	LM DUP-1052A	.	.	LM DUP-18627	.
Carcass Cooler Door Handle	.	.	.	.	.	.
Tempering Room Floor	.	.	.	.	.	.
Tempering Room Drain	.	.	.	.	.	.
Tempering Room Sink	.	.	.	.	.	.
Tempering Room to Fab Door	.	.	.	.	.	.
Tempering Room to Office Door	.	.	.	.	.	.
Tempering Room Lug Cart Wheels	.	.	.	LW AT27	.	.
Tempering Room Apron	.	.	.	.	.	.
Grinding to Retail Door	.	.	LW AT69	LW AT27	.	.
Grinding Area Floor	.	.	.	.	.	.
Grinding Area Drain	.	LM DUP-1052A	LM DUP-1052A	LI AT19	.	.
	.	.	LW AT69	.	.	.
Underside of Vacuum Pkger Lid	.	.	.	.	.	.

Vacuum Pkger Lid and Controls	.	.	.	.	.	.
Box Cooler Door	.	.	.	.	.	.
Staging Area Floor	.	.	LI AT38	.	.	LM DUP-1042B
Zuber Stuffer Non-Contact Surfaces	.		.	.	.	.
Raw/In-Process/Finished Areas-Annex Building						
Vestibule Drain	.	.	.	.	.	.
Preparation Area Drain	LM DUP-1052A	LM DUP-1052A	.	.	.	.
Drying Room Floor	.	LM DUP-1052A	.	.	.	.
Preparation Area to Vestibule Door	.	.	.	.	.	.
Preparation Area Floor	NS	.	.	NS	NS	NS
Preparation Area Hand Sink	NS	.	.	.	NS	NS
Preaparation Area Door to Brine Room	NS	.	.	.	NS	NS
Vestibule Floor	NS	NS	.	.	NS	NS
In-Process/Finished Product Areas						
Fab Area Cart Wheels	.	.	.	LW AT27	.	.
Fab Area Hand Sink	.	LM DUP-1052A	.	LW AT27	.	.

[a] NS indicates a site that was not sampled during a given sample collection and a "-" indicates that a sample did not test positive for *Listeria* during a given sample collection. *Listeria* positive samples are described by two capital letters to indicate the species that was isolated from the sample; where LM indicates *L. monocytogenes*, LI indicates *Listeria innocu*a, HLI indicates hemolytic *L. innocua*, and LW indicates *L. welshimeri*. *L. monocytogenes* molecular subtype is denoted (e.g., *Eco*RI ribotype DUP-1052A) and the *Listeria* spp. *sigB* allelic type (e.g., AT23) is denoted also denoted following species designation.

TABLE 2.6. *Listeria monocytogenes* and *Listeria spp.* contamination profiles for Plant 3 over a one-year period with bi-monthly sample collections.

Plant 3						
Sample Location	Dates Sampled					
	3/2007	5/2007	7/2007	9/2007	11/2007	1/2008
Raw/In-Process/Finished Areas						
Pipe from Refrigerator Unit/Floor Drain	.	.	.	.	.	.
Vacuum Packager Handle	.	.	.	.	HLI AT87	.
Weighing Scale Screen	.	.	.	.	HLI AT87	.
Employee Apron	.	.	.	.	.	.
Cooler Door Outside Handle	.	.	.	.	HLI AT87	.
Inside Vacuum Packager Board and Seal Bar	.	.	NS	.	.	.
RTE Packager Table Leg Extension	NS	NS	.	NS	NS	.
Floor Drain Near Vacuum Packager	NS	NS	NS	NS	NS	.
Door Handle and Light Switch in Break Room	NS	NS	NS	NS	NS	.
Handwash Sink Soap Dispenser Top	NS	NS	NS	NS	NS	.
Wall Seam near RTE Packager	NS	NS	NS	NS	NS	.
Riser Below Riser near RTE Packager	NS	NS	NS	NS	NS	.
Silicone Seal Below Cooler Door in RTE Area	NS	NS	NS	NS	NS	HLI AT87
Cracked Wall Panel Next to Office	NS	NS	NS	NS	NS	LM DUP-1061A
Door Frame Near Break Room Door	NS	NS	NS	NS	NS	.
Rubber Mat at Packaging Area	NS	NS	NS	NS	NS	LM DUP-1061A

Raw/Finished Areas						
Floor Drain Inside Cooler	.	.	.	.	HLI AT87	HLI AT87
Packaged Product Lugs in Cooler	.	.	.	.	.	.
RTE Product Lugs in Stand-in Cooler	.	.	.	.	.	.
Cooler Door Inside Handle	.	.	.	.	.	.

[a] NS indicates a site that was not sampled during a given sample collection and a "-" indicates that a sample did not test positive for *Listeria* during a given sample collection. *Listeria* positive samples are described by two capital letters to indicate the species that was isolated from the sample; where LM indicates *L. monocytogenes*, LI indicates *Listeria innocu*a, HLI indicates hemolytic *L. innocua*, and LW indicates *L. welshimeri*. *L. monocytogenes* molecular subtype is denoted (e.g., *Eco*RI ribotype DUP-1052A) and the *Listeria* spp. *sigB* allelic type (e.g., AT23) is denoted also denoted following species designation.

TABLE 2.7. *Listeria monocytogenes* and *Listeria spp.* contamination profiles for Plant 4 over a one-year period with bi-monthly sample collections.

Plant 4						
Sample Location	Dates Sampled					
	3/8/07	5/30/07	8/8/07	9/19/07	11/14/07	1/15/08
Raw/In-Process/Finished Areas						
Scale Face in RTE/Raw Area	.	.	.	.	.	.
Table Top in Packaging Area	.	.	.	.	.	.
Outside Doorknob to Cooler	.	.	.	.	.	.
Cooler Door Cracked Seal	.	.	.	.	.	.
Hand Wash Sink Soap Dispenser	.	.	.	.	.	LM DUP-1025B
Cooler Light Switch	.	.	.	.	.	LW AT89
Floor Drain Across from Spice Room	.	.	.	.	.	LM DUP-1025B
Office Entry White Flange	.	.	.	.	.	LM DUP-1025B
Steel Door to Dry Storage Wall Crack	.	.	.	.	.	LM DUP-1025B
Floor Crack in RTE/Carcass Cooler	.	.	.	.	.	LM DUP-1025B
Edge of Wood Cabinet Above Wrap Table	.	.	.	.	.	.
Raw/Finished Areas						
White Pipe/Drain in Cooler	.	.	.	.	.	.
White Shelf in Corner Cooler		.	.	.	.	.
Brown Plastic Container Package	.	.	.	.	.	.
White Pipe Beside RTE Area	.	.	.	.	.	.

Inside Doorknob in Cooler	.	.	.	.	.	.
Finished Areas						
Office Scale Buttons	.	.	.	.	.	.
Lid of Vacuum Packager	.	.	.	.	.	.
Vacuum Packager Buttons	.	.	.	.	.	.

[a] NS indicates a site that was not sampled during a given sample collection and a "-" indicates that a sample did not test positive for *Listeria* during a given sample collection. *Listeria* positive samples are described by two capital letters to indicate the species that was isolated from the sample; where LM indicates *L. monocytogenes*, LI indicates *Listeria innocu*a, HLI indicates hemolytic *L. innocua*, and LW indicates *L. welshimeri*. *L. monocytogenes* molecular subtype is denoted (e.g., *Eco*RI ribotype DUP-1052A) and the *Listeria* spp. *sigB* allelic type (e.g., AT23) is denoted also denoted following species designation.

TABLE 2.8. *Listeria monocytogenes* and *Listeria spp.* contamination profiles for Plant 5 over a one-year period with bi-monthly sample collections.

Plant 5						
Sample Location	Dates Sampled					
	3/8/07	5/30/07	8/8/07	9/19/07	11/14/07	1/15/08
Raw/In-Process/Finished Areas						
Pallet Jack Wheels	NS	LW AT69	NS	NS	NS	NS
Worker's Shoes	.	NS	NS	NS	NS	NS
Entrance Door Handle-Outside	.	NS	NS	NS	NS	NS
Room Entrance Door	.	NS	NS	NS	NS	NS
In-Process Areas						
Screw Leg of 3660 (Cooking Device) Under Panel 6	NS	NS	.	.	.	.
Chain Separating Pack and Cook	NS	NS	.	.	.	.
Wall and Floor in Cook Room	NS	NS	.	.	.	.
3660 Cooking Device-Panel 12	NS	NS	.	.	.	.
Floor Cookside	.	.	NS	NS	NS	NS
In-Process/Finished Areas						
Curve Belt Used To Transfer Product	.	.	.	.	.	.
Transfer Bar Between Curve Belt and Individual Quick Freeze	.	NS	NS	.	NS	NS
Wheel of Transfer Belt from Individual Quick Freeze to Packaging	NS	NS	.	LM DUP-1062A	.	.
Squeegee in Packaging Room	NS	NS	LM DUP-1056A	LM DUP-1062D	.	.
Individual Quick Freeze Belt	LW AT69	.	.	.	.	.

Wheel of Blue Tote Cart in Packaging Room	NS	NS	.	.	.	.
Weish Pack	NS	NS	.	.	.	.
Blue Tubs	NS	NS	.	.	.	.
Miscellaneous Table Packside	.	NS	NS	NS	.	.
Employee Gloves	.	.	.	.	.	.
Leg of Repack Table	NS	NS	.	.	.	.
Curve of Conveyor Frame	NS	.	NS	.	.	.
Wall on Packside	NS	NS	.	.	.	.
Packside Drain	NS	.	.	LM DUP-1053A	.	.
Transfer from 3660	.	.	.	.	.	.
Cook side drain	NS	.	.	.	.	.
Individual Quick Freeze Exhaust/Roof	NS	.	.	.	.	.
Packaging Floor Exit	.	NS	NS	NS	NS	NS
Koppens belt	.	.	NS	NS	NS	NS
Floor Under Koppens Belt	NS	.	NS	NS	NS	NS
Entrance Door Handle Inside	.	NS	NS	NS	NS	NS
Packaging Room Floor	.	NS	.	.	.	.
Individual Quick Freeze Exit Trap	NS	.	NS	NS	NS	NS
Black Mats Packside Drain	.	.	.	LM DUP-1053A	.	.
Incline conveyor	.	.	NS	NS	NS	NS

[a] NS indicates a site that was not sampled during a given sample collection and a "-" indicates that a sample did not test positive for *Listeria* during a given sample collection. *Listeria* positive samples are described by two capital letters to indicate the species that was isolated from the sample; where LM indicates *L. monocytogenes*, LI indicates *Listeria innocu*a, HLI indicates hemolytic *L. innocua*, and LW indicates *L. welshimeri*. *L. monocytogenes* molecular subtype is denoted (e.g., *Eco*RI ribotype DUP-1052A) and the *Listeria* spp. *sigB* allelic type (e.g., AT23) is denoted also denoted following species designation.

TABLE 2.9. *Listeria monocytogenes* and *Listeria spp.* contamination profiles for Plant 6 over a one-year period with bi-monthly sample collections.

Plant 6						
Sample Location	Dates Sampled					
	3/2007	6/2007	7/2007	10/2007	12/2007	1/2008
Raw/In-Process Areas						
Smokehouse Room Trough Floor Drain	LI AT70	LI AT31	LI AT70	.	LI AT70	.
Floor at Smokehouse Door	.	.	LI AT71	LI AT70	.	.
Floor Smokehouse Joint at South Smokehouse	.	.	.	.	.	LI AT53
Trench Floor Drain by South Smokehouse	LW AT16	.	LI AT70	LI AT23	.	LI AT53
Raw/In-Process/Finished Areas						
Smokehouse Truck Wheels	.	.	.	LI AT31	.	.
Packaging Room Squeegee	.	NS	NS	NS	NS	NS
Squeegee for Entire Plant Floors	NS	LI AT31	LI AT70	.	LI AT31	.
In-Process Areas						
Cooler Floor Drain by South Smokehouse	.	.	.	.	.	.
Cooler Door by South Smokehouse	.	.	.	.	.	.
Doors to Packaging Storage	.	.	.	.	.	.
Processed/Finished Areas						
Glove of Packaging Worker	.	NS	NS	NS	NS	NS
Cutting Board in Packaging Room	.	NS	NS	NS	NS	NS
Rallston Packaging Machine Loading Area	.	.	.	.	.	.

Metal Smokehouse Screen in Product Cooler	.	.	.	.	.	.
Plastic Smokehouse Screen in Product Cooler	.	.	.	.	NS	NS
Tabletop Under Cutting Board	.	.	.	.	.	.
Sliding Door Packaging to Meat Cooler	.	.	.	.	.	.
Trench Floor Drain in Packaging Room	.	LM DUP-1039C	LI AT70	.	.	.
Steel Table Leg by Floor Packager	.	LI AT70	.	.	.	.
Wheels to Slicer Cart in Packaging Area	.	LM DUP-1039C	LI AT70	NS	NS	NS
Slicer in Packaging Rooms	NS	.	NS	.	.	.
Table by Slicer	NS	.	.	.	.	.
Cooked Meat Cooler Floor Drain	LI AT70	LI AT31	NS	.	LI AT53	.
Frame of Metal Tree	NS	NS	NS	NS	.	.
Table Leg by Slicer	NS	NS	NS	LI AT70	.	.

[a] NS indicates a site that was not sampled during a given sample collection and a "-" indicates that a sample did not test positive for *Listeria* during a given sample collection. *Listeria* positive samples are described by two capital letters to indicate the species that was isolated from the sample; where LM indicates *L. monocytogenes*, LI indicates *Listeria innocu*a, HLI indicates hemolytic *L. innocua*, and LW indicates *L. welshimeri*. *L. monocytogenes* molecular subtype is denoted (e.g., *Eco*RI ribotype DUP-1052A) and the *Listeria* spp. *sigB* allelic type (e.g., AT23) is denoted also denoted following species designation.

FIGURE 2.1. Dendrograms of all *Listeria monocytogenes* isolates collected from each of the six plants tested over a one-year period.

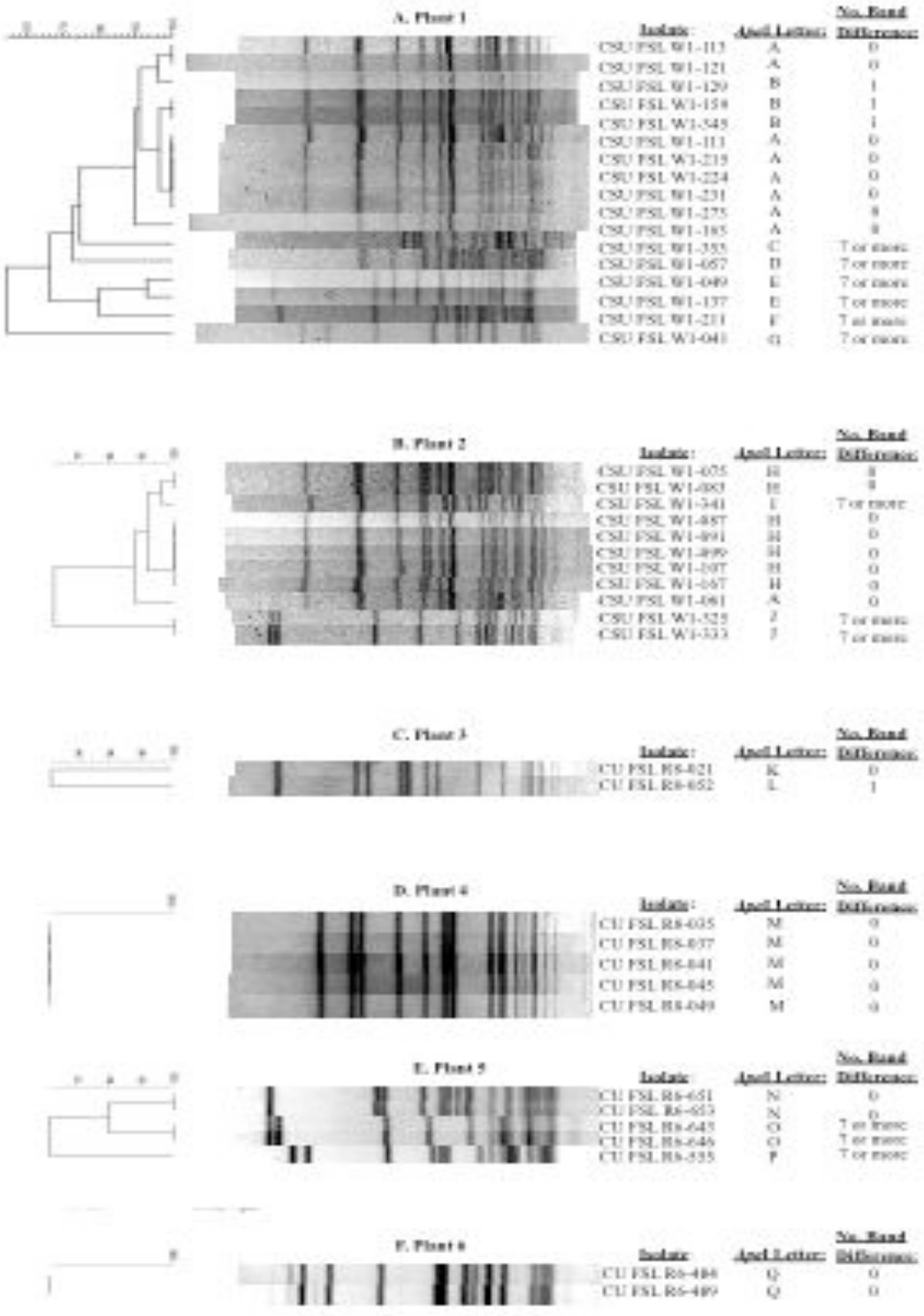

Figure 2.1. Dendrograms created using the unweighted pair group matching algorithm and the Dice correlation coefficient as implemented using BioNumerics software to visualize similarity of *L. monocytogenes* isolates from each RTE meat-processing plant. The first column following each PFGE patterns denotes the isolate identification (e.g., CSU FSL W1-041), the second column depicts the *Apa*I letter assignment given to that specific isolate (e.g., B) and the final column indicates the number of band differences between each isolate from the predominant PFGE subtype isolated from that specific plant. Dendrograms of *Apa*I digested DNA from *L. monocytogenes* isolates from plant 1-6 are depicted by Figs 1A-F, respectively.

CHAPTER III

A two-year longitudinal study on *Listeria* contamination patterns in small and very small ready-to-eat meat processing plants: impact of in-plant training sessions

Abstract

A two year longitudinal study was conducted to (i) probe *Listeria* contamination patterns in six small or very small RTE meat processing plants through combined testing and molecular subtyping and (ii) control *Listeria* through real-time communication of testing results and in-plant *Listeria* training sessions. A total of 688 environmental sponge samples were collected from non-food contact surfaces in year 1 and a total of 1,055 samples were collected in year 2, including non-food contact surfaces along with food contact surfaces and finished RTE meat products for some plants. Samples were screened following a modified version of the U. S. Food Safety and Inspection Service *L. monocytogenes* BAX® PCR test and microbiologically analyzed to confirm the presence of viable *L. monocytogenes* and to detect other *Listeria* spp. *L. monocytogenes* was isolated from 5.1% of samples, where the prevalence of this pathogen ranged from 0.9% to 8.8% across different plants. *Listeria* spp. other than *L. monocytogenes* were isolated from 6.5% of samples with the prevalence of other *Listeria* spp. ranging from 0.8% to 11.4% across different plants. Testing results were communicated to plants immediately after they became available and in-plant training sessions involving all plant employees

were conducted in between year 1 and 2. *Listeria* contamination was significantly lower ($P < 0.0001$) and *L. monocytogenes* contamination was marginally reduced ($P = 0.1079$) in year 2 as compared to year 1. Comparison of pre- and post-training evaluations indicated that employees across all plants significantly ($P = 0.0001$) increased their knowledge of *Listeria* ecology and control. The findings highlight the importance of a routine combined *Listeria* testing and molecular subtyping program, real-time communication of testing results and in-plant training sessions to control *Listeria* in the plant environment.

3.1. Introduction

Listeria monocytogenes is a facultative intracellular pathogen that may cause invasive disease in susceptible host populations, including the elderly, pregnant women, neonates and otherwise immuno-compromised individuals. Clinical manifestations of invasive listeriosis include septicemia, encephalitis, meningitis, and late-term spontaneous abortions in pregnant women (Schlech, 2000). Although listeriosis is a relatively rare infectious disease, *L. monocytogenes* infections are associated with notably high hospitalization (90%) and case fatality (20-30%) rates as compared to other foodborne pathogens (Mead et al., 1999). *L. monocytogenes* infections account for approximately 30% of all fatalities due to known foodborne pathogens each year in the U.S. (Mead et al., 1999). The current *L. monocytogenes* risk assessment identified RTE deli meats as the overwhelmingly most common food responsible for human listeriosis, as 1,599 out of 1,798 median estimated cases per annum (nearly 90%) were predicted to be attributed to deli meats (U.S. FDA/CFSAN, USDA/FSIS, and CDC, 2003). Detection

of *L. monocytogenes* in RTE foods along with human listeriosis outbreaks and sporadic listeriosis cases have severe negative economic consequences for food processors and the U.S. economy, including costs attributed to product recalls, lawsuits, hospitalizations and deaths (Ivanek et al., 2004a).

Control of *Listeria* represents a particular challenge for the ready-to-eat (RTE) food industry due to the common presence and persistence of *Listeria* in virtually all environments along the food continuum (e.g., Gray et al., 2004; Lappi et al., 2004; Nightingale et al., 2004; Sauders et al., 2006a). *Listeria* is a plant saprophyte as the organism thrives at the soil-plant interface (Beuchat, 1999). *L. monocytogenes* survives well and is able to persist outside of a mammalian host and commonly is isolated from environmental samples such as water, soil, and vegetation (Fenlon, 1999). Weiss and Seeliger (1975) isolated *L. monocytogenes* from up to 44% of plant and surface soil samples from agricultural fields. A more recent study showed *L. monocytogenes* to be present in 1.3% of soil, water, and vegetation samples from pristine environments (i.e., national parks and wildlife feeding grounds) and 7.3% of samples from urban environments, clearly indicating that *L. monocytogenes* is present in the general environment (Sauders et al., 2006a). *L. monocytogenes* also is commonly shed in the feces of healthy cattle (approximately 25% of asymptomatic cattle excrete *L. monocytogenes* in their feces) and can be isolated from approximately 20% of animal feedstuff along with farm environmental samples (i.e., soil and water) (Nightingale et al., 2004). Because *Listeria* is common-place in the general environment and livestock production environments, this organism also is a frequent contaminant of raw materials

used for food processing (e.g., raw meat) and thus it is reasonable to assume *Listeria* will be routinely introduced into the food processing plant environment.

Previous studies demonstrated that *L. monocytogenes* is difficult to control and may persist in the food processing plant environment (e.g., Autio et al., 1999; Chasseignaux et al., 2001; Destro et al., 1996; Ho et al., 2007; Lappi et al., 2004; Kabuki et al., 2004; Miettinen et al., 1999; Peccio et al., 2003; Senczek et al., 2000; Tompkin, 2002; Tompkin, 1992) and that a diversity of *L. monocytogenes* subtypes can be found in food processing plant (e.g., Lappi et al., 2004). For example, in a two-year longitudinal study, Lappi et al. (2004) isolated 22 different *L. monocytogenes* subtypes (*Eco*RI ribotypes) from four seafood processing plants. Furthermore, individual seafood processing plants appeared to be persistently contaminated by one or more subtypes and the same subtypes that persisted in the plant environment also were implicated in the majority of finished RTE seafood product contamination events. While *L. monocytogenes* is the only human pathogen in the *Listeria* genus of public health significance (Liu, 2006) the molecular ecology and contamination patterns of other *Listeria* spp. in the food processing plant environment also is relevant as other *Listeria* spp. may be used to indicate potential *L. monocytogenes* harborage sites and elucidate *L. monocytogenes* transmission (Tompkin, 2002).

We conducted a two-year longitudinal study that included bi-monthly sampling of finished RTE meat products, food contact surfaces and non-food contact surfaces from six small or very small RTE meat plants in Colorado, Kansas and Nebraska (i) identify *Listeria* harborage sites and persistent strains in small or very small RTE meat processing plants through combined testing and molecular subtyping, including potential routes of

finished RTE product contamination and (ii) control *Listeria* persistence and transmission through real-time communication of testing results and in-plant *Listeria* training sessions. Results from this study highlight the importance of routine combined testing and molecular subtyping, communication of testing results and training sessions for all plant employees to convey basic knowledge regarding *Listeria* ecology, listeriosis, along with controlling *Listeria* in the RTE meat plant environment.

3.2. Materials and Methods

Sample Collection. Two small, (10 to <500 employees) or very small (<10 employees or <2.5 million dollars in annual sales) RTE meat processing plants each located in Colorado, Kansas and Nebraska were enrolled in a longitudinal study to probe *Listeria* contamination patterns. Plant-specific demographics and production characteristics were described in our previous study (Williams et al., 2010 *In press*). A total of 1,743 samples were collected from these six plants over twelve bimonthly sample collections beginning in February, 2007, through January, 2009. Samples were collected from 10 to 25 non-food contact surfaces (e.g., walls, doors, drains, floors, sinks, cart wheels, and equipment surfaces), 5 to 8 food contact surfaces (e.g. tables, slicers, cutting boards, knives), and up to ten finished RTE meat product (e.g. hot dogs, bratwurst, frankfurters, bologna) samples from a given plant during each of the six sample collection visits. The Whirl-Pak® Speci-Sponge® kit bag (Nasco, Fort Atkinson, Wis.) was used for environmental and food contact surface sample site collections in which size of the swabbing area should be 30 cm by 30 cm whenever possible. Sponges were held on ice during transit to the laboratory and processed within 24 h of collection. Packaged

RTE meat product samples were held collected from the finished product holding cooler and held on ice during transit back to the laboratory where two 25 g samples of each food product's outside surface were aseptically collected using a sterile scalpel in a biosafety cabinet and placed in a sterile Whirl-Pak® bag.

Bacteriological analysis. A modified version of the U.S. Food Safety and Inspection Service procedure for detection of *L. monocytogenes* (USDA:FSIS MLG) was used for initial PCR screening and subsequent microbiological analyses of all 1,743 environmental, food contact and finished RTE meat product samples. Briefly, environmental sponges samples were aseptically transferred into a filter Whirl-Pak® stomacher bag (Seward Ltd., London, United Kingdom) and environmental and food samples were homogenized with 225 ml of University of Vermont Medium (UVM) broth using a stomacher (Stomacher 400; Seward Ltd., West Sussex, UK) and incubated at 30°C for 24 h. The overnight enrichment was streaked onto modified Oxford medium (MOX) and incubated at 35°C for a minimum of 24 h, and the enriched sample also was used to inoculate a secondary morpholinepropanesulfonic acid-buffered *Listeria* enrichment broth (MOPS-BLEB) at a 1:100 dilution. After incubation at 35°C for 24 h, the secondary MOPS-BLEB enrichment was used to prepare DNA template for screening using the BAX® *L. monocytogenes* kit (DuPont Qualicon, Wilmington, DE). An aliquot of the secondary MOPS-BLEB enrichment was streaked onto MOX and incubated as described above. Melt curve analysis was used to screen each sample for the presence of amplification of a PCR internal positive control (IPC) and a gene fragment that is unique to *L. monocytogenes*. The ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster, CA) or iQ5 Multicolor RT PCR Detection System (Bio-Rad,

Hercules, CA) were used for real-time cycling and subsequent melt curve analyzes. Melt curves were analyzed for a peak around 78°C (indicating amplification of the IPC) and a second peak at approximately 84-85°C (indicating amplification of a gene fragment unique to *L. monocytogenes*). To culturally confirm each sample that was positive for the screening PCR and/or showed colonies with typical *Listeria* morphology on MOX plates from the primary enrichment, up to four *Listeria*-like colonies were sub-streaked onto *Listeria monocytogenes* Plating Medium (LMPM; Biosynth Biochemica & Synthetica, Naperville, IL.). On LMPM media, *L. monocytogenes* and *L. ivanovii* colonies appear as a turquoise-blue color due to hydrolysis of a colorimetric phospholipase substrate, while all other non-pathogenic *Listeria* spp. form white colonies on LMPM plates. *Eco*RI ribotyping was used confirm presumptive *L. monocytogenes* colonies. Up to four confirmed colonies that were confirmed as *L. monocytogenes* and up to two colonies presumed to belong to *Listeria* spp. other than *L. monocytogenes* were sub-streaked for purity on brain heart infusion (BHI; Becton Dickson, Sparks, MD) agar plates, cultivated overnight in BHI broth and preserved at –80°C in 15% glycerol.

sigB Sequence Typing. All presumptive *Listeria spp.* were further characterized by PCR amplification and DNA sequence typing of a stress response gene *sigB* as detailed in our previous study (Nightingale et al., 2005). Amplicons were sequenced and assembled and BLAST searches were performed for each *sigB* sequence and all *sigB* sequence were aligned using MegaAlign software (DNAstar, Madison, WI) and assigned a *sigB* allelic type (Rozas et al., 2003). A previous study showed that *sigB* sequencing was able to speciate *Listeria* spp. isolates in concordance with biochemical confirmation by the API

Listeria test (Sauders et al., 2004). Sequence data for each isolate can be found on PathogenTracker at (www.pathogentracker.net).

Molecular Serotyping. A previously described multiplex PCR (Doumith et al., 2004) was used to assign all *L. monocytogenes* isolates into molecular serogroups each containing one of the four serotypes associated with the majority of human disease (e.g. 1/2a, 1/2b, 1/2c, and 4b). This assay detects serotype-specific markers (i.e., *lmo*0737, *lmo*1118, ORF2819 and ORF2110) identified through comparative microarray experiments and a fragment of *prs* that is conserved in all *Listeria spp.* as a PCR amplification control. *L. monocytogenes* isolates belonging to each major human disease associated serotype (i.e., 1/2a, 1/2b, 1/2c, and 4b), as determined by conventional slide agglutination serotyping, were used as controls in each multiplex PCR reaction.

Ribotyping. *Eco*RI ribotyping using the RiboPrinter® Microbial Characterization System was performed to confirm and subtype a single *L. monocytogenes* isolate from each presumptive positive sample (DuPont-Qualicon, Wilmington, DE) as detailed previously by Bruce et al. (1996). Briefly, bacterial cells were lysed to release total bacteria DNA followed by digestion with *Eco*RI. Resultant DNA fragments then were size separated via electrophoresis and captured on a membrane. The membrane was treated with a labeled *Escherichia coli* rRNA operon probe, which only hybridizes DNA fragments containing rRNA genes, exposed to a substrate followed by visualization of hybridized fragments using a charge coupled camera device. Resultant fragment pattern data were normalized using custom software and assigned a DuPont ID (e.g., DUP-1039). If isolate patterns differed by only a slight shift of one band from previously denoted DuPont ID's an extra letter was used (e.g., DUP-1039B and DUP-1039C) to differentiate

isolates and patterns that were not previously observed were assigned a uniqe DuPont ID. Ribotype patterns are accessible through PathogenTracker (www.pathogentracker.net).

Pulsed Field Gel Electrophoresis (PFGE) typing. Pulsed-field gel electrophoresis typing of a single *L. monocytogenes* isolate from each positive sample was performed following the Centers for Disease Control and Prevention standardized PulseNet protocol (CDC, 2001). Briefly, agarose plugs were made by suspending an optimal optical density of a bacterial cultures from the lawn of an overnight BHI plate in cell lysis buffer and imbedding an aliquot of the suspension in 1% SeaKem Gold® agarose (Cambrex Bio Science, Rockland, ME). Agarose plug slices were lysed overnight, washed, and digested separately with *Asc*I for 5.5 h at 37°C and *Apa*I for 7 h at 30°C. Agarose plug slices then were loaded into 1.5% agarose gels and electrophoresed on a CHEF Mapper XA (BioRad Laboratories) for 21 h with switch times of 4.0 s to 40.0 s. *Xba*I digested *Salmonella* ser. Braenderup (H9812) DNA was used as a reference size standard (Hunter et al., 2005). Agarose gels were stained in ethidium bromide and resultant images were captured. PFGE gels were analyzed by similarity clustering analyses using the unweighted pair group matching algorithm and the Dice correlation coefficient as implemented using BioNumerics software (Applied Maths; Saint-Matins-Latem, Belgium). All PFGE patterns were uploaded into the PathogenTracker database (www.pathogentracker.net).

Communication of Results and In-plant Training Sessions. After each sample collection, testing results were communicated to each plant through written documentation as soon as they became available. Upon completion of sample collection and testing in year 1 and molecular characterization of *Listeria* isolates an in-plant

training session for all employees was conducted at each plant enrolled in the study. The in-plant training session was led by senior personnel involved in this project and included information regarding *Listeria* ecology and transmission, human listeriosis, *Listeria* contamination patterns and control in the RTE food processing plant (i.e., cleaning and sanitation). Testing and molecular subtyping results for the first year of the project were communicated to all plant employees through the in-plant training session. The impact of the training session was assessed through administration of both a pre- and a post-training questionnaire, which included 23 questions about *Listeria* and its control.

Statistical Analysis. A paired T-test was used to compare (i) the prevalence of *Listeria* (i.e., all *Listeria* spp.) and *L. monocyotgenes* observed in year 1 and 2 across all six plants and (ii) pre- and post-training scores for all employees across all six plants as implemented through the TTEST procedure in Statistical Analyses Software (SAS: Cary, NC).

3.3. Results

Plant 1. Based on twelve bi-monthly sample collections over a two-year period, 8.8% of 422 samples from plant 1 tested positive for *L. monocytogenes* and 9.7% of samples contained *Listeria* spp. other than *L. monocytogenes* (Table 3.1). While the majority of *L. monocytogenes* isolates from plant 1 were classified into the molecular serogroup containing 1/2b, 3b and 7, a few isolates fell into other molecular serogroups, including those containing serotypes 1/2a and 3a; 1/2c and 3c; and 4b, 4d, and 4e (Table 3.2). A single *L. monocytogenes* isolate from each of the 37 positive samples from plant 1 was characterized by *Eco*RI ribotyping. *L. monocytogenes* isolates from plant 1 were

classified into nine distinct ribotypes, where the majority of isolates from this plant (n=22) belonged to ribotype DUP-1052A (Tables 3.2 and 3.3). Seven isolates belonged to ribotype 116-239-S-2, two isolates belonged to ribotype DUP-18602, and a single isolate each belonged to ribotypes DUP-1042B, DUP-18616, DUP-1044A and DUP-1042C in the raw/in-process areas of this plant. In addition, an isolate belonging to ribotype DUP-1048A was detected on the packaging area floor and a single isolate belonging to ribotype DUP-1030A was isolated from a finished RTE product produced by plant 1 (Table 3.3). *L. monocytogenes* isolates belonging to ribotype DUP-1052A were isolated from plant 1 on six of the 12 sample collections and this ribotype was detected on more than one occasion in six sample sites. The majority of *L. monocytogenes* positive sites were detected in the raw/in-process areas, except during the second sample collection in which ribotype DUP-1052A was detected in multiple samples from the finished product area on the second sampling and the ninth sample collection in which this strain was detected on a food contact surface (Table 3.3). Further characterization of the 37 *L. monocytogenes* isolates from plant 1 by PFGE typing revealed 27 different combined ApaI/AscI pulsotypes (Table 3.2). We created dendrograms to visualize clustering of isolates within each plant based on *Apa*I restriction digests to identify the predominant strain(s) within each plant and to assess the relatedness of other strains within the same plant to the predominant strain(s). In plant 1, 12 isolates belonged to a predominant *Apa*I PFGE type A (Figure 3.1); this predominant *Apa*I PFGE pattern was used as the reference type in order to classify other isolates as closely related (three or fewer band difference), possibly related (four to six band difference) or distantly related (more than seven band difference) as compared to the

predominant strain according to the criteria specified by Tenover et al. (1995). Based on this analysis, nine isolates were closely related to the predominant *Apa*I pattern and 16 isolates were not related to the most common *Apa*I pattern observed among isolates from plant 1 (Figure 3.1). These results support that strains that are closely related to the predominant subtype may emerge within the environment of a given plant over time.

BLAST searches of *sigB* sequences were performed to confirm and speciate other *Listeria* spp. isolates. BLAST search results showed that *L. innocua* and *L. welshimeri* were present in the environment of plant 1. Unique combinations of *sigB* polymorphisms were used to assign specific allelic types to *Listeria* spp. isolates to probe the molecular ecology of other *Listeria* spp. in each plant enrolled in this study. *Listeria* spp. isolates from plant 1 grouped into six different *sigB* allelic types. *L. innocua* isolates belonging to allelic type 56 (AT56) were predominant in the environment of plant 1 (n=32), followed by *L. welshimeri* AT19 isolates (Table 3.3). In addition, a single isolate each belonged to *L. welshimeri* AT27, *L. welshimeri* AT69, *L. innocua* AT109 and *L. innocua* AT110. *Listeria* spp. other than *L. monocytogenes* were only isolated from raw/in-process environmental sites over the duration of the study. The same allelic type (*L. innocua* AT56) was isolated on more than one occasion from six sample collection sites. Interestingly, *L. innocua* AT56 was isolated from the floor of the stuff area from the drain of the stuff area on seven separate sample collections (Table 3.3). In addition, *L. innocua* AT56 was isolated at least twice from four other sites (e.g. raw area apron, chop/grind area drain, raw area smoketruck wheels and smokehouse drain) in the raw area. Two specific sites in the raw area, including the chopper controls and door between the stuff

area and smokehouse, tested positive for *L. welshimeri* AT19 on two different sample collections (Table 3.3).

Plant 2. Results from environmental testing showed that *L. monocytogenes* was isolated from 4.4% and *Listeria* spp. other than *L. monocytogenes* were isolated from 3.2% of 315 samples from plant 2 (Table 3.1). Of the 14 *L. monocytogenes* isolates from plant 2, twelve isolates belonged to the molecular serogroup containing serotypes 1/2b, 3b, and 7, while two isolates were classified into a molecular serogroup that includes serotypes 1/2a and 3a (Table 3.2). Characterization of *L. monocytogenes* isolates from plant 2 by *Eco*RI ribotyping revealed three ribotypes, where eleven isolates belonged to a predominant ribotype (DUP-1052A) and two isolates were identified as ribotype DUP-18627 and one isolate as DUP-1042B (Tables 3.2 and 3.4). A *L. monocytogenes* isolate belonging to ribotype DUP-1052A was isolated during five of the twelve sampling collections. During the sample collection on 5/17/07, ribotype DUP-1052A was isolated from six different sites located within both buildings comprising plant 2 (Table 3.4). Ribotype DUP-1052A was isolated on two sequential sample collections from two drains in the raw/in-process area of plant 2 (i.e., the grinding area and preparation area drains), suggesting that *L. monocytogenes* persisted in the drains of this plant. Pulsed field gel electrophoresis typing based on both *Apa*I and *Asc*I restriction grouped *L. monocytogenes* isolates from plant 2 into six different pulsotypes (Table 3.2). The majority of the *Listeria monocytogenes* isolates grouped within the same *Apa*I pulsotype (*Apa*I pulsotype O) and three of the remaining *Apa*I pulsotypes (*Apa*I pulsotypes P, Q, and R) were possibly related to the predominant *Apa*I pulsotype, as determined by a four to six band difference from the predominant strain (Figure 3.1). The other two isolates belonging to

*Apa*I pulsotype S showed a greater than seven band difference from pulsotype O and thus were distantly related to the predominant pulsotype in plant 2 (Figure 3.1). *Listeria spp.* other than *L. monocytogenes* were isolated during four of the 12 samplings, speciated and assigned allelic types using *sigB* sequence data. Interestingly, the predominant species of *Listeria* detected in plant 2 was *L. welshimeri* and only three *L. innocua* isolates were identified (Table 3.4). Four *sigB* allelic types were found overall with *L. welshimeri* AT27 representing the most common *sigB* allelic type, which was isolated from four different sample locations during one sample collection (Table 3.4). *Listeria* spp. other than *L. monocytogenes* did not appear to persist in the environment of plant 2 as only *L. innocua* AT38 was isolated from the environment of plant 2 on more than one occasion.

Plant 3. Only two samples tested positive for *L. monocytogenes* (0.9%), while 22 other *Listeria* spp. isolates (9.4%) were detected from 234 samples collected from plant 2 over the duration of the study (Table 3.1). The two *L. monocytogenes* isolates could not be assigned into a molecular serogroup responsible for the majority of human disease (Table 3.2). Both, *L. monocytogenes* isolates were collected during year 1 and belonged to ribotype DUP-1061A, a ribotype that includes isolates belonging to *L. monocytogenes* genetic lineage III (Wiedmann et al., 1997) and lineage III isolates are not typeable by the molecular serotyping multiplex PCR employed here (Nightingale et al., 2007). Both *L. monocytogenes* isolates belonged to the same combined *Apa*I/*Asc*I pulsotype (Table 3.2). Analyses of *sigB* sequences revealed that plant 3 also harbored *L. innocua*, *L. welshimeri* and a rare hemolytic *L. innocua* strain (Sauders et al., 2006). This hemolytic *L. innocua* strain was isolated from the environment of plant 2 on four different sample collections and was isolated from three sample sites (e.g. silicone seal in cooler door, rubber mat at

packaging area, and the floor drain inside the cooler) on more than one occasion. In addition, eight *L. innocua* AT31 isolates were collected over five continuous samplings with two specific sites being colonized by this same allelic type on multiple visits (i.e., an apron worn by the same employee and the floor drain inside the cooler).

Plant 4. Over the two-year sampling, a total of 227 samples were collected from plant 4 with 7.1% testing positive for *L. monocytogenes* and 2.6% positive for *Listeria* spp. other than *L. monocytogenes* (Table 3.1). *L. monocytogenes* was detected during six of the 12 sample collections and 15 of the 16 *L. monocytogenes* isolates belonged to the same molecular serogroup (1/2b, 3b, 7) and ribotype (DUP-1025B) (Table 3.2). The remaining *L. monocytogenes* isolate belonged to the molecular serogroup including serotypes 1/2a and 3a and was classified as ribotype DUP-1062A (Table 3.2). Three sample collection sites were colonized by ribotype DUP-1025B over multiple samplings (e.g. floor drain across from spice room, floor crack in RTE carcass cooler, and metal threshold on doorway by wash sink) (Table 3.6). Combined *Apa*I/*Asc*I PFGE characterization showed a total of six pulsotypes (Table 3.2) and all but one isolate belonged to the same *Apa*I restriction pulsotype (Figure 3.1). Cluster analyses of *Apa*I patterns showed that only one *L. monocytogenes* isolate was distantly related to the predominant pulsotype U (Figure 3.1). *Listeria* spp. other than *L. monocytogenes* isolates were detected during five of the 12 samplings. Three isolates detected on three separate sample collections were characterized as *L. welshimeri* AT89. A single isolate each classified as *L. innocua* AT109, *L. innocua* AT6 and *L. innocua* AT11 also were detected (Table 3.6).

Plant 5. *L. monocytogenes* was detected in 3.1% of a total of 256 samples from collected from plant 5, while two samples (0.8%) were found to be positive for *Listeria* spp. other

than *L. monocytogenes* (Table 3.1). *L. monocytogenes* were detected during three of the 12 sampling collections and seven of the eight *L. monocytogenes* isolates from plant 5 belonged to the same molecular serogroup, which contains serotypes 1/2a and 3a (Table 3.2). Ribotying results from plant 5 showed that half of the *L. monocytogenes* isolates belonged to ribotype DUP-1053A, which was found over two samplings, and the remaining *L. monocytogenes* isolates belonged to unique ribotypes (e.g. DUP-1056A, DUP-1062A, DUP-1062D, and DUP-1052A) that were each observed once (Table 3.7). Further characterization of the *L. monocytogenes* isolates by PFGE typing found six different pulsotypes based on combined *Apa*I and *Asc*I restriction (Table 3.2). There was no evidence of a predominant *Apa*I pulsotype within plant 5 (Figure 3.1). s*igB* allelic typing showed that the two other *Listeria* spp. isolates from the first two samplings belonged to *L. welshimeri* AT 69 (Table 3.7).

Plant 6. *L. monocytogenes* was detected in 4.2% and *Listeria* spp. other than *L. monocytogenes* was found in 11.4% of 289 samples collected from plant 6 (Table 3.2). *L. monocytogenes* was isolated from the environment of plant 6 during five of the 12 samplings and in finished RTE product during the final sample collection (Table 3.8). All environmental *L. monocytogenes* isolates belonged to the same molecular serogroup (i.e., serotypes 1/2c and 3c) and the finished product isolate to fell within the molecular serogroup that includes serotypes 4b, 4d and 4e (Table 3.2). All eleven of the *L. monocytogenes* isolates from the environment of plant 6 belonged to ribotype (DUP-1039C) and the finished RTE product isolate belonged to ribotype DUP-1044A. PFGE characterization of the twelve *L. monocytogenes* isolates by *Apa*I and *Asc*I restriction revealed three unique combined enzyme pulsotypes (Table 3.2). Cluster analyses of *Apa*I

restriction patterns showed that nine isolates clustered within a predominant *Apa*I pulsotype (pulsotype BB; Figure 3.1), while three isolates differed from the predominant pulsotype by a greater than seven band difference indicating no relatedness to the predominant pulsotype (Figure 3.1). The same *L. monocytogenes* strain (ribotype DUP-1039C) was detected in two environmental sites on more than one occasion (i.e., smokehouse room trough floor drain and trench floor drain in packaging room) (Table 3.8). *sigB* sequencing of all other *Listeria* spp. isolates in plant 6 revealed the presence of seven different allelic types, where *L. innocua* AT70 was the predominant allelic type with 14 isolates followed by *L. innocua* AT53 containing 10 isolates. Five *Listeria* spp. isolates were classified as *L. innocua* AT31, and one each were grouped into *L. welshimeri* AT16, *L. innocua* AT23, *L. innocua* AT71, and *L. welshimeri* AT43 (Table 3.8). Four environmental sites were colonized by the same *sigB* allelic type over multiple sample collections, including two drains and two floor sites within the raw/in-process areas in plant 6 (Table 3.8).

Impact of In-Plant Training Sessions. An in-plant training session for all employees at each of the six plants enrolled in the study was performed in between year 1 and year 2 sampling to provide all plant employees with fundamental knowledge regarding listeriosis, *Listeria* ecology, and transmission and control of *Listeria* in the RTE meat processing plant environment. The impact of these training sessions was assessed through administration of the same set of 23 questions before and after the training session. We observed a significant increase ($P < 0.0001$) in employee post-training test scores across all six plants as compared to pre-training test scores (Figure 3.2). We also observed a significantly ($P < 0.0001$) lower prevalence of *Listeria* spp. other than *L.*

monocytogenes and marginally ($P = 0.1079$) reduced prevalence of *L. monocytogenes* across all six plants in year 2 following the in-plant training sessions as compared to year 1 (Table 3.1). Augmenting plant employee knowledge regarding listeriosis, *Listeria* ecology, transmission and its control may partially explain the reduced prevalence of *Listeria* observed in environment of plants in year 2 compared to year 1.

3.4. Discussion

Six small or very small RTE meat plants were enrolled in a two-year longitudinal study to probe *Listeria* contamination patterns and the impact of a routine testing and molecular subtyping program along with in-plant training sessions on *Listeria* ecology and transmission for all employees. Results from this study showed that (i) a routine combined testing and molecular subtyping program can elucidate *Listeria* contamination patterns, which appear to be unique to each plant, and identify harborage sites and (ii) real-time communication of testing results and in-plant training sessions on *Listeria* ecology and transmission may lead to enhanced control of *Listeria* contamination in the plant environment. Findings from this study highlight the utility of routine testing for *L. monocytogenes* and other *Listeria* spp. coupled with molecular subtyping to elucidate the unique molecular *Listeria* ecology and transmission dynamics within a given plant and the impact of real-time communication of testing results and in-plant training sessions to control *Listeria*. Combined routine testing and subtyping can elucidate plant specific contamination patterns, including identification of harborage sites. Over the two-year sampling period each of the six small or very small RTE meat plants enrolled in this study showed

different *Listeria* contamination patterns. For example, plants 1 and 2 were characterized by a similar number of samples that testing positive for *L. monocytogenes* and for *Listeria* spp. other than *L. monocytogenes*. On the other hand plants 3 and 6 were characterized by a higher prevalence of other *Listeria* spp. as compared to *L. monocytogenes*, while plants 4 and 5 showed a higher prevalence of *L. monocytogenes* as compared other *Listeria* spp. (Table 3.1). Although all samples were analyzed to detect both *L. monocytogenes* and other *Listeria* spp., only a few samples contained both *L. monocytogenes* and *Listeria* spp. other than *L. monocytogenes* (Tables 3.3-3.8). Similar to previous studies on *Listeria* contamination patterns in large RTE meat processing plants (Tompkin 2002; Tompkin et al., 1992), results from this study support that the relationship between the presence of *L. monocytogenes* and other *Listeria* spp. in the environment of small or very small plants is not necessarily consistent. The utility of testing for *Listeria* spp. as an indicator of *L. monocytogenes* contamination appears to depend on the unique *Listeria* ecology observed for each plant. Regardless of whether or not the presence of *L. monocytogenes* and other *Listeria* spp. is consistent across different plants or within a given plant, detection of any *Listeria* represents a sanitation failure and a potential harborage site that should be targeted for aggressive cleaning and sanitation. For the most part, *Listeria* contamination was contained within the raw/in-process areas within each plant. Overall, plants 1 and 6 had the highest number of environmental samples that tested positive for either *L. monocytogenes* and other *Listeria* spp., where plant 1 had 78 samples testing positive for *Listeria* and plant 2 had 45 samples testing positive for *Listeria*. It is worth noting that plants 1 and 6 also had a finished RTE meat product contamination event during the course of the project, supporting that a higher

overall prevalence of *Listeria* in the plant environment may be a risk factor for cross-contamination of finished RTE products.

Molecular characterization of a single *L. monocytogenes* and/or other *Listeria* spp. isolate from each *Listeria* positive sample provided even greater insight into plant-specific *Listeria* contamination, including identification of persistent strains and harborage sites. For example, two *L. monocytogenes* strains (i.e., ribotypes DUP-1052A and 116-239-S-2) and a *L. innocua* strain (AT56) were the predominant *Listeria* strains isolated from the environment of plant 1 (Table 3.3). *L. monocytogenes* DUP-1052A, *L. monocytogenes* 116-239-S-2 and *L. innocua* AT56 were isolated from the environment of plant 1 on six, four and nine occasions, respectively. Interestingly, plant 1 received raw meat for processing from two different suppliers, which might explain the continuous re-introduction into and predominance of two *L. monocytogenes* strains in the raw/in-process area in the environment of this plant. The floor and drain in the room where sausage was stuffed into casings represented particularly problematic *Listeria* harborage sites in the environment of plant 1, as *Listeria* (often belonging to one of the predominant strains listed above) was isolated from the floor and drain in the stuffing area during all 12 and 11 out of 12 sample collections, respectively. *L. monocytogenes* isolates belonging to ribotype DUP-1052A and *L. welshimeri* AT27 isolates were most commonly isolated from the environment within plant 2, where drains, cart wheels, floors, a door and a hand-sink were shown to be contaminated by *Listeria* on multiple occasions (Table 3.4). Plant 3 was predominantly colonized by an atypical hemolytic *L. innocua* strain (*sigB* allelic type 87), which was isolated repeatedly from stress mats in the packaging area and in the finished product cooler (i.e., seal under the door and floor)

during more than one sample collection. Interestingly, a rare genetic lineage III *L. monocytogenes* strain (ribotype DUP-1061A; Wiedmann et al., 1997) also was detected in two samples from the environment of plant 2 during one sample collection.

Combined testing and molecular subytping results from plant 4 indicated that this plant was predominantly colonized by a single *L. monocyotgenes* strain (ribotype DUP-1025B), which persisted in the environment of plant 4 over six consecutive bi-monthly sample collections. Drains, floor cracks and low spots on the floor capable of accumulating standing water represented *L. monocytogenes* harborage sites of particular concern in plant 4 (Table 3.6). On the other hand, plant 5 appeared to be characterized by transient *Listeria* contamination as the plant was not colonized by a predominant *L. monocytogenes* strain (based on combined ribotyping and PFGE characterization) or any other predominant strain belonging to another *Listeria* spp. (Table 3.6). The squeegee used to clean the floor in the finished product packaging room; however, tested positive for a different *L. monocytogenes* strain on three separate occasions. In plant 6, the majority of *Listeria* positive samples from year 1 contained *Listeria* spp. other than *L. monocytogenes*; however, the frequency of *L. monocytogenes* contamination events increased in the second year of sampling. All *L. monocytogenes* isolates from plant 6 belonged to the same ribotype (DUP-1039C), which seemed to persist in the drains of this plant and sporadically contaminate environmental sites in the finished packaging area that were adjacent to or located near finished RTE product contact surfaces (e.g., leg of table by packager, wheels of slicer cart and table by slicer). Multiple strains belonging to other *L. innocua* (i.e., AT53 and AT70) also persisted in the drains and on the floors of the raw/in-process area of plant 6 over the two year period. Also, two different *Listeria*

spp. *sigB* allelic types (*L. innocua* AT31 and *L. innocua* AT70) were isolated from the squeegee used to clean the floors of the entire plant on more than one occasion, indicating a possible hazard for transmission of this bacterium from raw/in-process areas to finished product areas (Table 3.8).

The results support that each small or very small RTE meat plant enrolled in this study appeared to be characterized by a unique *Listeria* ecology; however, certain environmental sites in general (e.g., drains, floors and stress mats) across plants may be particularly likely to harbor *Listeria* and other equipment in the plant (e.g., cart wheels and squeegees) also can become persistently contaminated by *Listeria* posing a risk for transmission to finished product handling areas. Results from this study are consistent with a previous two-year longitudinal study that used combined testing and subtyping to monitor *Listeria* contamination patterns in four cold smoked seafood processing plants, where each plant was colonized by a single or few predominant *L. monocytogenes* strain(s) and certain sites across all four plants appeared to harbor *Listeria* (e.g., drains, stress mats and cart wheels) (Lappi et al., 2004). Taken together, this study and previous studies highlight the critical need for stringent cleaning and sanitation procedures to eliminate *Listeria* harborage sites within the plant environment and reduce the risk of *Listeria* transmission to finished RTE foods.

Communication of testing results and in-plant training sessions may facilitate enhanced control of *Listeria* contamination and transmission in the plant environment. Microbiological culture results were communicated to plant owners or managers immediately after they become available following each sample collection. In addition, in-plant training sessions for all plant employees in each plant were conducted

upon completion of the first year of sampling and molecular subtyping of *Listeria* isolates from year 1 to impart general knowledge on listeriosis, *Listeria* ecology and transmission along with control of *Listeria* in the RTE meat plant environment. Interestingly, we observed a significant increase in employee knowledge across all six plants regarding *Listeria* following the in-plant training sessions along with a reduced prevalence of *Listeria* in the environment of the plants enrolled in this study in year two as compared to year one. To our knowledge this is the first report describing a possible association between real-time communication of testing results along with instructing *Listeria* training sessions for all plant employees a observed reduction in the prevalence of *Listeria* in the environment of small and very small *Listeria* plants. It also is noteworthy that a routine combined testing and molecular subtyping program can be instrumental in the identification of *Listeria* harborage sites and transmission patterns that may present a high risk for finished RTE product contamination.

3.5. Conclusion

Each small or very small RTE meat plant enrolled in this two-year longitudinal study was characterized by a unique *Listeria* ecology, including a range of observed *L. monocytogenes* and other *Listeria* spp. prevalence in the environment of each plant over time. The presence of *L. monocytogenes* and other *Listeria* spp. was consistent from some but not all plants and our results support that an overall increased prevalence of *Listeria* in the plant environment indicates inadequate cleaning and sanitation and may be a risk factor for finished RTE meat contamination events. Combined testing and molecular subtyping proved useful in identification of *Listeria* strains that were endemic

or sporadic in the environment of each plant. In addition, molecular subtyping lead to the identification of persistent strains and associated harborage sites along with contaminated equipment that may have facilitated the transmission of *Listeria* through the plant environment. Finally, communicating results from routine testing along with in-plant training sessions may be useful tools to increase fundamental knowledge regarding *Listeria* and may lead to the enhanced control of this bacterium in the RTE meat plant environment.

3.6. References

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TABLE 3.1. Summary of *Listeria* prevalence in small and very small ready-to-eat meat processing plants

	No. samples positive for *L. monocytogenes* (% positive)		No. samples positive for other *Listeria* spp. (% positive)[a]		Year 1 and Year 2 combined no. samples positive for *L. monocytogenes* (% positive)	Year 1 and Year 2 combined no. samples positive for other *Listeria* spp. (% positive)[a]
Ready-to-eat meat processing plant	[#] *P<0.1079*		[*] *P<0.0001*			
	Year 1	Year 2	Year 1	Year 2	Year 1 and Year 2	Year 1 and Year 2
Plant 1	17 (10.8)	20 (7.6)	25 (15.9)	16 (6.0)	37(8.8)	41(9.7)
Plant 2	11 (6.9)	3 (1.9)	8 (5.0)	2 (1.3)	14(4.4)	10(3.2)
Plant 3	2 (2.9)	0 (0.0)	7 (10.0)	15 (9.2)	2(0.9)	22(9.4)
Plant 4	5 (7.9)	10 (6.2)	1 (1.5)	5 (3.1)	5(7.1)	6(2.6)
Plant 5	5 (4.4)	3 (2.1)	2 (1.7)	0 (0.0)	8(3.1)	2(0.8)
Plant 6	2 (1.7)	10 (5.9)	22 (18.3)	11 (6.5)	12(4.2)	33(11.4)
All plants	42 (6.1)	46 (4.4)[#]	65 (9.5)	49 (4.6)[*]	88(5.1)	114(6.5)

TABLE 3.2. Summary of molecular characteristics of a single *Listeria monocytogenes* isolate selected to represent each *L. monocytogenes* positive sample.

Isolate	ApaI pulsotype	AscI pulsotype	Combined ApaI/AscI pulsotype	EcoRI ribotype[a]	Molecular serogroup[b]	Plant	Sample collection
CSUFSL W1-159	B	P	1	DUP-1052A	1/2b, 3b or 7	Plant 1	3
CSUFSL W1-211	L	A	2	DUP-18616	1/2a or 3a	Plant 1	4
CSUFSL W1-215	A	J	3	DUP-1052A	1/2b, 3b or 7	Plant 1	4
CSUFSL W1-224	A	J	3	DUP-1052A	1/2b, 3b or 7	Plant 1	4
CSUFSL W1-231	A	J	3	DUP-1052A	1/2b, 3b or 7	Plant 1	4
CSUFSL W1-273	A	K	4	DUP-1052A	1/2b, 3b or 7	Plant 1	5
CSUFSL W1-345	B	J	5	DUP-1052A	1/2b, 3b or 7	Plant 1	6
CSUFSL W1-111	A	K	6	DUP-1052A	1/2b, 3b or 7	Plant 1	2
CSUFSL W1-113	A	K	6	DUP-1052A	1/2b, 3b or 7	Plant 1	2
CSUFSL W1-163	A	P	7	DUP-1052A	1/2b, 3b or 7	Plant 1	3
CSUFSL W1-121	A	O	8	DUP-1052A	1/2b, 3b or 7	Plant 1	2
CSUFSL W1-129	A	O	8	DUP-1052A	1/2b, 3b or 7	Plant 1	2
CSUFSL W1-353	E	T	9	DUP-1052A	1/2b, 3b or 7	Plant 1	6
CSUFSL W1-057	F	Q	10	DUP-1042B	1/2b, 3b or 7	Plant 1	1
CSUFSL W1-049	J	R	11	DUP-18602	1/2a or 3a	Plant 1	1
CSUFSL W1-137	J	S	12	DUP-18602	1/2a or 3a	Plant 1	2

CSUFSL W1-041	N	U	13	DUP-1048A	1/2c or 3c	Plant 1	1
CSUFSL W1-495	D	J	14	DUP-1052A	1/2b, 3b or 7	Plant 1	9
CSUFSL W1-499	A	H	15	DUP-1052A	1/2b, 3b or 7	Plant 1	9
CSUFSL W1-519	G	E	16	116-239-S-2	1/2b, 3b or 7	Plant 1	10
CSUFSL W1-527	F	C	17	116-239-S-2	1/2b, 3b or 7	Plant 1	10
CSUFSL W1-535	G	F	18	116-239-S-2	1/2b, 3b or 7	Plant 1	10
CSUFSL W1-555	A	I	19	DUP-1052A	1/2b, 3b or 7	Plant 1	12
CSUFSL W1-563	F	D	20	116-239-S-2	1/2b, 3b or 7	Plant 1	12
CSUFSL W1-571	A	J	3	DUP-1052A	1/2b, 3b or 7	Plant 1	12
CSUFSL W1-579	C	J	21	DUP-1052A	1/2b, 3b or 7	Plant 1	12
CSUFSL W1-587	F	D	22	116-239-S-2	1/2b, 3b or 7	Plant 1	12
CSUFSL W1-595	A	J	3	DUP-1052A	1/2b, 3b or 7	Plant 1	12
CSUFSL W1-387	H	L	23	116-239-S-2	1/2b, 3b or 7	Plant 1	7
CSUFSL W1-401	I	N	24	DUP-1044A	4b, 4d or 4e	Plant 1	8
CSUFSL W1-405	L	B	25	DUP-1030A	1/2a or 3a	Plant 1	8
CSUFSL W1-451	D	J	14	DUP-1052A	1/2b, 3b or 7	Plant 1	9
CSUFSL W1-459	D	J	14	DUP-1052A	1/2b, 3b or 7	Plant 1	9
CSUFSL W1-467	K	G	26	116-239-S-2	1/2b, 3b or 7	Plant 1	9
CSUFSL W1-475	D	J	14	DUP-1052A	1/2b, 3b or 7	Plant 1	9
CSUFSL W1-483	D	J	14	DUP-1052A	1/2b, 3b or 7	Plant 1	9
CSUFSL W1-491	M	M	27	DUP-1042C	1/2b, 3b or 7	Plant 1	9

CSUFSL W1-061	Q	Y	28	DUP-1052A	1/2b, 3b or 7	Plant 2	1
CSUFSL W1-075	O	X	29	DUP-1052A	1/2b, 3b or 7	Plant 2	2
CSUFSL W1-083	O	X	29	DUP-1052A	1/2b, 3b or 7	Plant 2	2
CSUFSL W1-087	O	X	29	DUP-1052A	1/2b, 3b or 7	Plant 2	2
CSUFSL W1-091	O	X	29	DUP-1052A	1/2b, 3b or 7	Plant 2	2
CSUFSL W1-099	O	X	29	DUP-1052A	1/2b, 3b or 7	Plant 2	2
CSUFSL W1-107	O	X	29	DUP-1052A	1/2b, 3b or 7	Plant 2	2
CSUFSL W1-167	O	X	29	DUP-1052A	1/2b, 3b or 7	Plant 2	3
CSUFSL W1-341	P	W	30	DUP-1042B	1/2b, 3b or 7	Plant 2	6
CSUFSL W1-325	S	V	31	DUP-18627	1/2a or 3a	Plant 2	5
CSUFSL W1-333	S	V	31	DUP-18627	1/2a or 3a	Plant 2	5
CSUFSL W1-415	R	X	32	DUP-1052A	1/2b, 3b or 7	Plant 2	8
CSUFSL W1-423	R	X	32	DUP-1052A	1/2b, 3b or 7	Plant 2	8
CSUFSL W1-511	O	X	33	DUP-1052A	1/2b, 3b or 7	Plant 2	9
CSUFSL R8-021	T	Z	34	DUP-1061A	Untypeable	Plant 3	6
CUFSL R8-052	T	Z	34	DUP-1061A	Untypeable	Plant 3	6
CUFSL R8-035	U	DD	35	DUP-1025B	1/2b, 3b or 7	Plant 4	6
CUFSL R8-037	U	DD	35	DUP-1025B	1/2b, 3b or 7	Plant 4	6
CUFSL R8-041	U	DD	35	DUP-1025B	1/2b, 3b or 7	Plant 4	6
CUFSL R8-045	U	DD	35	DUP-1025B	1/2b, 3b or 7	Plant 4	6
CUFSL R8-049	U	DD	35	DUP-1025B	1/2b, 3b or 7	Plant 4	6

CUFSL R8-425	V	BB	36	DUP-1062A	1/2a or 3a	Plant 4	7
CUFSL R8-430	U	FF	37	DUP-1025B	1/2b, 3b or 7	Plant 4	7
CUFSL R8-434	U	FF	37	DUP-1025B	1/2b, 3b or 7	Plant 4	7
CUFSL R8-1202	U	EE	38	DUP-1025B	1/2b, 3b or 7	Plant 4	8
CUFSL R8-1206	U	EE	38	DUP-1025B	1/2b, 3b or 7	Plant 4	8
CUFSL R8-1899	U	EE	38	DUP-1025A	1/2b, 3b or 7	Plant 4	9
CUFSL R8-1907	U	CC	39	DUP-1025A	1/2b, 3b or 7	Plant 4	9
CUFSL R8-1910	U	CC	39	DUP-1025A	1/2b, 3b or 7	Plant 4	9
CUFSL R8-2139	U	CC	39	DUP-1025A	1/2b, 3b or 7	Plant 4	10
CUFSL R8-2141	U	CC	39	DUP-1025A	1/2b, 3b or 7	Plant 4	10
CUFSL R8-2615	U	AA	40	DUP-1025A	1/2b, 3b or 7	Plant 4	11
CUFSL R6-651	Y	GG	41	DUP-1053A	1/2a or 3a	Plant 5	4
CUFSL R6-653	Y	HH	42	DUP-1053A	1/2a or 3a	Plant 5	4
CUFSL R6-643	Z	II	43	DUP-1062A	1/2a or 3a	Plant 5	4
CUFSL R6-646	Z	II	43	DUP-1062D	1/2a or 3a	Plant 5	4
CUFSL R6-555	W	JJ	44	DUP-1056A	1/2a or 3a	Plant 5	3
CUFSL R8-875	X	LL	45	DUP-1052A	1/2b, 3b or 7	Plant 5	7
CUFSL R8-879	AA	KK	46	DUP-1053A	1/2a or 3a	Plant 5	7
CUFSL R8-2420	AA	KK	46	DUP-1053A	1/2a or 3a	Plant 5	7
CUFSL R6-484	DD	OO	47	DUP-1039C	1/2c or 3c	Plant 6	2
CUFSL R6-489	DD	OO	47	DUP-1039C	1/2c or 3c	Plant 6	2

CUFSL R8-1315	BB	MM	48	DUP-1039C	1/2c or 3c	Plant 6	8
CUFSL R8-1318	BB	MM	48	DUP-1039C	1/2c or 3c	Plant 6	8
CUFSL R8-1323	BB	MM	48	DUP-1039C	1/2c or 3c	Plant 6	8
CUFSL R8-1329	BB	MM	48	DUP-1039C	1/2c or 3c	Plant 6	8
CUFSL R8-1912	BB	MM	48	DUP-1039C	1/2c or 3c	Plant 6	9
CUFSL R8-2147	BB	MM	48	DUP-1039C	1/2c or 3c	Plant 6	10
CUFSL R8-2742	BB	MM	48	DUP-1039C	1/2c or 3c	Plant 6	11
CUFSL R8-2746	BB	MM	48	DUP-1039C	1/2c or 3c	Plant 6	11
CUFSL R8-2748	BB	MM	48	DUP-1039C	1/2c or 3c	Plant 6	11
CUFSL R8-3377	CC	NN	49	DUP-1044A	4b, 4d or 4e	Plant 6	12

[a] When an assigned DuPont ID included more than one distinct ribotype pattern (e.g., patterns differing by a single weak band), each pattern was designated with an additional alphabetized letter (e.g., DUP-1039A and DUP-1039B)
[b] Isolates were classified into a molecular serogroup using a multiplex PCR assay previously described by Doumith et al. (2004).

TABLE 3.3. *Listeria monocytogenes* and *Listeria spp.* contamination profiles for Plant 1 over a one-year period with bi-monthly sample collections.

Plant 1												
Sample Location	Dates Sampled											
	3/8/07	3/8/07	5/30/07	8/8/07	9/19/07	11/14/07	3/8/08	5/30/08	7/8/08	9/19/08	11/14/08	1/15/09
Raw/In-Process Area												
Pallet Jack Handle	.	.	.	.	.	.	.	.	.	.	.	.
Raw Area to Packaging Area Door	.	.	.	.	LI AT56	.	.	.	LM 116-239-S-2	.	.	.
Raw Area Ice Machine	.	.	LM DUP-1052A	.	LI AT56	LM DUP-1052A	.	.	.	.	.	.
Time Clock	NS	NS	NS	.	NS	NS	.	.	.	.	.	.
Grind/Chop Area Floor	.	.	.	LM DUP-1052A	LI AT56	.	.	.	LM DUP-1052A	.	.	LM DUP-1052A
Raw Area Hand Sink	.	.	.	.	LI AT56	.	.	.	LM DUP-1042C	.	.	.
Raw Area Apron	.	.	LI AT56	.	LI AT19	.	LI AT56	.	LM DUP-1052A	LM 116-239-S-2	.	LM DUP-1052A
									LI AT56			
Raw Area Chopper Controls	.	.	LI AT19	.	LI AT19	LM DUP-1052A	.	.	LI AT56	.	.	.
Chop/Grind Area Drain	.	LW AT27	LI AT56	.	LM DUP-1052A	.	LM 116-239-S-2	.	LI AT56	.	.	LM DUP-1052A
Stress Mat in Brine Area	NS	NS	.	NS	NS	NS	.	.	.	.	.	.
Stuff Area Floor	LM DUP-1042B	LW AT69	LM DUP-1052A	LM DUP-1052A	LI AT56	LI AT56	LI AT56	LI AT56	LM DUP-1052A	LM 116-239-S-2	LI AT56	LM DUP-1052A
	.	.	LI AT56	.	.	.	.	.	.	.	.	LI AT56
Raw Area Smktruck Wheels	.	.	LI AT56	.	LI AT56	.						

Stuff Area Drain	LM DUP-18602	.	LI AT56	LM DUP-1052A	LI AT56	LI AT56	.LI AT56	LI AT56	LM DUP-1052A	LM 116-239-S-2	LI AT56	LM 116-239-S-2
												LI AT56
Stuff Area to Smoke Door	.	LI AT109	LI AT19	.	LI AT19	.	.	LM DUP-1044A	LI AT56	.	.	LM 116-239-S-2
Door to Packaging Area	.	.	.	.	.	.	.	.		.	.	.
Smokehouse to Packaging Door	.	.	.	LM DUP-18616	.	.	.	.	LM DUP-1052A	.	.	.
Shower Area Drain	.	.	.	.	.	.	-	.	.LI AT56	.	.	-
Smokehouse Drain	.	LI AT110	.	.	LI AT56	.	.LI AT56	.	.	.	.	LI AT56
Smokehouse #1 Door Handle	.	LM DUP-18602	.	.	.	.	.	.	.	.	.	.
	.	LI AT56	.	.	.	.	.	.	.	.	.	.
Smokehouse # 2 Handle	NS	NS	.	NS	NS	NS	NS	NS	NS	NS	NS	NS
Smokehouse Control Panel	NS	NS	NS	.	NS	NS	NS	NS	NS	NS	NS	NS
Finished Product Area												
Packaging Area Drain #1	.	.	.	.	.	.	.	.	.	.	.	.
Packaging Area Drain #2	NS	.	NS	NS	NS	NS	.	.	.	.	.	.
Packaging to Cooler Door	.	.	.	.	.	.	.	.	.	.	.	.
Smoketruck Wheels	.	LM DUP-1052A	.	.	.	.	.	.	.	.	.	.
Packaging to Dock Door	.	LM DUP-1052A	.	.	.	.	.	.	.	.	.	.
Vacuum Packager Lid and Controls	.	.	.	.	.	.	.	.	.	.	.	.

Packaging Area Hand Sink	.	LM DUP-1052A	.	.	.	.	.	.	.	.	.	.
Packaging Area Floor	LM DUP-1048A	.	.	.	.	.	.	.	.	.	.	.
Cooler Box Cart Wheels	NS	NS	NS	.	NS	NS	.	.	.	.	.	.
Cooler Floor	.	LM DUP-1052A	.	.	.	.	.	.	.	.	.	.
Cooler to Dock Door	NS	NS	NS	.	NS	NS	.	.	.	.	.	.
Food Contact Surfaces												
Table 1	NS	NS	NS	NS	NS	NS	.	.	.	.	.	
Table 2	NS	NS	NS	NS	NS	NS	.	.	.	.	.	
Gloves of workers at table 1	NS	NS	NS	NS	NS	NS	.	.	.	.	.	
Gloves of workers at table 2	NS	NS	NS	NS	NS	NS	.	.	.	.	.	
Knife at table 1	NS	NS	NS	NS	NS	NS	.	.	LM DUP-1052A	.	.	
Knife at table 2	NS	NS	NS	NS	NS	NS	.	.	.	.	.	
Finished Product												
RTE Finished Product	NS	NS	NS	NS	NS	NS	.	LM DUP-1030A	.	.	.	.

[a] NS indicates a site that was not sampled during a given sample collection and a "-" indicates that a sample did not test positive for *Listeria* during a given sample collection. *Listeria* positive samples are described by two capital letters to indicate the species that was isolated from the sample; where LM indicates *L. monocytogenes*, LI indicates *Listeria innocu*a, HLI indicates hemolytic *L. innocua*, and LW indicates *L. welshimeri*. *L. monocytogenes* molecular subtype is denoted (e.g., *Eco*RI ribotype DUP-1052A) and the *Listeria* spp. *sigB* allelic type (e.g., AT23) is denoted also denoted following species designation.

TABLE 3.4. *Listeria monocytogenes* and *Listeria spp.* contamination profiles for Plant 1 over a one-year period with bi-monthly sample collections.

Plant 2												
Sample Location	Dates Sampled											
	3/20/07	3/20/07	5/17/07	8/9/07	9/17/07	11/14/07	1/15/08	3/20/07	3/20/07	5/17/07	8/9/07	9/17/07
Raw/In-Process Areas-Main Facility Building												
Shipping Dock Table	.	.	.	.	.	.	.	.	.	.	.	.
Drip Area Floor	.	LM DUP-1052A	.	.	LM DUP-18627	.	.	.	.	.	.	.
Drip Area Drain	.	LM DUP-1052A	.	.	LM DUP-18627	.	.	.	.	.	.	.
Carcass Cooler Door Handle	.	.	.	.	.	.	.	.	.	.	.	.
Tempering Room Floor	.	.	.	.	.	.	.	.	.	.	.	.
Tempering Room Drain	.	.	.	.	.	.	.	.	.	.	.	.
Tempering Room Sink	.	.	.	.	.	.	.	LI AT38	.	.	.	.
Tempering Room to Fab Door	.	.	.	.	.	.	.	.	.	.	.	.
Tempering Room to Office Door	.	.	.	.	.	.	.	.	.	.	.	.
Tempering Room Lug Cart Wheels	.	.	.	LW AT27	.	.	.	LM DUP-1052A	.	LW AT69	.	.
Tempering Room Apron	.	.	.	.	.	.	.	.	.	.	.	.
Grinding to Retail Door	.	.	LW AT69	LW AT27	.	.	.	.	.	.	.	.
Grinding Area Floor	.	.	.	.	.	.	.	LM DUP-1052A	.	.	.	.

Grinding Area Drain	.	LM DUP-1052A	LM DUP-1052A	LI AT19	.	.	.	.	.	.	.	.
	.	.	LW AT69	.	.	.	.	.	.	.	.	.
Underside of Vacuum Pkger Lid	.	.	.	.	.	.	.	.	.	.	.	.
Vacuum Pkger Lid and Controls	.	.	.	.	.	.	.	.	.	.	.	.
Box Cooler Door	.	.	.	.	.	.	.	.	.	.	.	.
Staging Area Floor	.	.	LI AT38	.	.	LM DUP-1042B	.	.	.	.	.	.
Zuber Stuffer Non-Contact Surfaces	.		.	.	.	.	.	.	.	.	.	.
Raw/In-Process/Finished Areas-Annex Building												
Vestibule Drain	.	.	.	.	.	.	.	.	.	.	.	.
Preparation Area Drain	LM DUP-1052A	LM DUP-1052A	.	.	.	.	.	.	.	.	.	.
Drying Room Floor	.	LM DUP-1052A	.	.	.	.	.	.	.	.	.	.
Preparation Area to Vestibule Door	.	.	.	.	.	.	.	.	.	.	.	.
Preparation Area Floor	NS	.	.	NS	NS	NS	.	.	.	.	.	.
Preparation Area Hand Sink	NS	.	.	.	NS	NS	.	.	.	.	.	.
Preaparation Area Door to Brine Room	NS	.	.	.	NS	NS	.	.	.	.	.	.

Vestibule Floor	NS	NS	.	.	NS	NS	.	.	.	.	.	.
In-Process/Finished Product Areas												
Fab Area Cart Wheels	.	.	.	LW AT27	.	.	.	.	.	.	.	.
Fab Area Hand Sink	.	LM DUP-1052A	.	LW AT27	.	.	.	.	LM DUP-1052A	.	.	.
Food Contact Surfaces												
Cutting Board	NS	NS	NS	NS	NS	NS	NS	.	.	.	.	.
Stainless Steel Table	NS	NS	NS	NS	NS	NS	NS	.	.	.	.	.
Meat Tote	NS	NS	NS	NS	NS	NS	NS	.	.	.	.	.
Finished Product												
RTE Finished Product	NS	NS	NS	NS	NS	NS	NS	.	NS	NS	NS	NS

[a] NS indicates a site that was not sampled during a given sample collection and a "-" indicates that a sample did not test positive for *Listeria* during a given sample collection. *Listeria* positive samples are described by two capital letters to indicate the species that was isolated from the sample; where LM indicates *L. monocytogenes*, LI indicates *Listeria innocu*a, HLI indicates hemolytic *L. innocua*, and LW indicates *L. welshimeri*. *L. monocytogenes* molecular subtype is denoted (e.g., *Eco*RI ribotype DUP-1052A) and the *Listeria* spp. *sigB* allelic type (e.g., AT23) is denoted also denoted following species designation.

TABLE 3.5. *Listeria monocytogenes* and *Listeria spp.* contamination profiles for Plant 1 over a one-year period with bi-monthly sample collections.

Plant 3												
Sample Location	Dates Sampled											
	3/2007	3/2007	5/2007	7/2007	9/2007	11/2007	3/2007	3/2007	5/2007	7/2007	9/2007	11/2007
Raw/In-Process/Finished Areas												
Pipe from Refrigerator Unit/Floor Drain	.	.	.	.	.	.	.	.	.	.	.	.
Vacuum Packager Handle	.	.	.	.	HLI AT87	.	.	NS	.	.	.	.
Weighing Scale Screen	.	.	.	.	HLI AT87	.	.	.	.	.	.	.
Employee Apron	.	.	.	.	.	.	LI AT31	LI AT31	.	.	.	.
Cooler Door Outside Handle	.	.	.	.	HLI AT87	.	.	.	.	.	.	.
Inside Vacuum Packager Board and Seal Bar	.	.	NS	.	.	.	.	.	.	.	.	.
RTE Packager Table Leg Extension	NS	NS	.	NS	NS	.	.	.	.	.	.	.
Floor Drain Near Vacuum Packager	NS	NS	NS	NS	NS	.	HLI AT87	.	.	.	.	.
Door Handle and Light Switch in Break Room	NS	NS	NS	NS	NS	.	HLI AT87	.	.	.	.	.
Handwash Sink Soap Dispenser Top	NS	NS	NS	NS	NS	.	.	.	.	.	.	.
Wall Seam near RTE Packager	NS	NS	NS	NS	NS	.	.	.	.	.	.	.
Riser Below Riser near RTE Packager	NS	NS	NS	NS	NS	.	.	.	.	.	.	.
Silicone Seal Below Cooler Door in RTE Area	NS	NS	NS	NS	NS	HLI AT87	HLI AT87	.	LI AT31	.	.	.
Cracked Wall Panel Next to Office	NS	NS	NS	NS	NS	LM DUP-1061A	.	.	.	LI AT71	.	.
Door Frame Near Break Room Door	NS	NS	NS	NS	NS	.	.	.	.	.	.	.
Rubber Mat at Packaging Area	NS	NS	NS	NS	NS	LM DUP-1061A	HLI AT87	.	LI AT31	HLI AT87	LW AT69	.
Raw/Finished Areas												

Floor Drain Inside Cooler	.	.	.	.	HLI AT87	HLI AT87	LI AT31	.	LI AT31	LI AT31	LI AT31	.
Packaged Product Lugs in Cooler	.	.	.	.		.	.	.	.	.	.	.
RTE Product Lugs in Stand-in Cooler	.	.	.	.	.	.	.	.	.	.	.	.
Cooler Door Inside Handle	.	.	.	.	.	.	.	.	.	.	.	.
Tsble with Unpacked RTE	NS	NS	NS	NS	NS	NS	.	.	NS	.	.	.
Gloves-Handling RTE Product	NS	NS	NS	NS	NS	NS	.	.	.	NS	.	.
Scale with RTE	NS	NS	NS	NS	NS	NS	.	NS	NS	.	.	.
Tabletop where packer RTE is placed	NS	NS	NS	NS	NS	NS	.	NS	.	NS	.	NS
Emloyee Handling RTE-apron	NS	NS	NS	NS	NS	NS	.	NS	NS	NS	NS	NS
Lug with Unpacked RTE	NS	NS	NS	NS	NS	NS	.	.	.	.	NS	NS
Scissors Cutting RTE	NS	NS	NS	NS	NS	NS	NS	.	.	.	NS	NS
Smokehouse Rack with RTE	NS	NS	NS	NS	NS	NS	NS	.	.	NS	NS	NS
Cooking Racks	NS	NS	NS	NS	NS	NS	NS	NS	NS	.	NS	NS
Knife Cutting RTE	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	.	NS
Vacuum Bag	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	.	NS
Cutting Board with RTE	NS	NS	NS	NS	NS	NS	NS	NS	NS	.	NS	.
Knife Blade Cutting RTE	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	.
RTE Smoker Rack	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	.

[a] NS indicates a site that was not sampled during a given sample collection and a "-" indicates that a sample did not test positive for *Listeria* during a given sample collection. *Listeria* positive samples are described by two capital letters to indicate the species that was isolated from the sample; where LM indicates *L. monocytogenes*, LI indicates *Listeria innocu*a, HLI indicates hemolytic *L. innocua*, and LW indicates *L. welshimeri*. *L. monocytogenes* molecular subtype is denoted (e.g., *Eco*RI ribotype DUP-1052A) and the *Listeria* spp. *sigB* allelic type (e.g., AT23) is denoted also denoted following species designation.

TABLE 3.6. *Listeria monocytogenes* and *Listeria spp.* contamination profiles for Plant 1 over a one-year period with bi-monthly sample collections.

Plant 4												
Sample Location	Dates Sampled											
	3/8/07	3/8/07	5/30/07	8/8/07	9/19/07	11/14/07	1/15/08	3/8/07	5/30/07	8/8/07	9/19/07	11/14/07
Raw/In-Process/Finished Areas												
Scale Face in RTE/Raw Area	.	.	.	.	.	.	.	.	.	.	.	.
Table Top in Packaging Area	.	.	.	.	.	.	.	.	.	.	.	.
Outside Doorknob to Cooler	.	.	.	.	.	.	LI AT109	.	.	.	.	.
Cooler Door Cracked Seal	.	.	.	.	.	.	.	.	.	.	.	.
Hand Wash Sink Soap Dispenser	.	.	.	.	.	LM DUP-1025B	.	.	.	.	.	.
Cooler Light Switch	.	.	.	.	.	LW AT89	.	.	.	.	.	.
Floor Drain Across from Spice Room	.	.	.	.	.	LM DUP-1025B	LM DUP-1025B	LM DUP-1025B	LW AT89	LM DUP-1025B	.	.
Office Entry White Flange	.	.	.	.	.	LM DUP-1025B	.	LW AT89	.	.	.	.
Steel Door to Dry Storage Wall Crack	.	.	.	.	.	LM DUP-1025B	.	.	.	.	.	.
Floor Crack in RTE/Carcass Cooler	.	.	.	.	.	LM DUP-1025B	LM DUP-1025B	LM DUP-1025B	LM DUP-1025B	.	LI AT11	.
Edge of Wood Cabinet Above Wrap Table							LM DUP-1025B		LM DUP-1025B	LM DUP-1025B	LM DUP-1025B	
Raw/Finished Areas												
White Pipe/Drain in Cooler	.	.	.	.	.	.	.	.	.	.	LI AT6	.
White Shelf in Corner Cooler		.	.	.	.	.		.	.	.	.	.
Brown Plastic Container Package	.	.	.	.	.	.	.	.	.	.	.	.
White Pipe Beside RTE Area	.	.	.	.	.	.	.	.	.	.	.	.

Inside Doorknob in Cooler	.	.	.	.	.	.	.	.	LM DUP-1025B	.	.	.
Finished Areas												
Office Scale Buttons	.	.	.	.	.	.	.	.	.	.	.	.
Lid of Vacuum Packager	.	.	.	.	.	.	.	.	.	.	.	.
Vacuum Packager Buttons	.	.	.	.	.	.	.	.	.	.	.	.
Food Contact Surfaces												
Gloves	NS	NS	NS	NS	NS	NS	.	.	.	NS	.	.
Lug Holding Product	NS	NS	NS	NS	NS	NS	.	.	.	.	.	.
Vacuum Packager Button Pressed w/ Gloved Hands	NS	NS	NS	NS	NS	NS	.	NS	NS	NS	NS	NS
Vacuum Packager Lid Where Glove Touches	NS	NS	NS	NS	NS	NS	.	NS	NS	NS	NS	NS
Butcher Wrap wher Product is Placed	NS	NS	NS	NS	NS	NS	.	NS	NS	NS	NS	NS
Inside of Vacuum Packager	NS	NS	NS	NS	NS	NS	NS	.	NS	NS	NS	NS
Tabletop Where Product is Stuffed	NS	NS	NS	NS	NS	NS	NS	.	.	.	NS	NS
Inside and Outside of Vacuum Bag	NS	NS	NS	NS	NS	NS	NS	.	NS	NS	NS	NS
Saw Top-Slicing RTE	NS	NS	NS	NS	NS	NS	NS	NS	NS	.	NS	NS
Knife used to Cut RTE	NS	NS	NS	NS	NS	NS	NS	NS	NS	.	NS	NS
Plastic RTE Wrap	NS	NS	NS	NS	NS	NS	NS	NS	NS	.	NS	NS
RTE Scissors	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	.	NS
RTE Bag	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	.	NS
RTE Tabletop	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	.

Finished Product												
RTE Finished Product	NS	NS	NS	NS	NS	NS	.	NS	.	NS	.	.

[a] NS indicates a site that was not sampled during a given sample collection and a "-" indicates that a sample did not test positive for *Listeria* during a given sample collection. *Listeria* positive samples are described by two capital letters to indicate the species that was isolated from the sample; where LM indicates *L. monocytogenes*, LI indicates *Listeria innocu*a, HLI indicates hemolytic *L. innocua*, and LW indicates *L. welshimeri*. *L. monocytogenes* molecular subtype is denoted (e.g., *Eco*RI ribotype DUP-1052A) and the *Listeria* spp. *sigB* allelic type (e.g., AT23) is denoted also denoted following species designation.

TABLE 3.7. *Listeria monocytogenes* and *Listeria spp.* contamination profiles for Plant 1 over a one-year period with bi-monthly sample collections.

Plant 5												
Sample Location	Dates Sampled											
	3/8/07	3/8/07	5/30/07	8/8/07	9/19/07	11/14/07	1/15/08	3/8/07	5/30/07	8/8/07	9/19/07	.
Raw/In-Process/Finished Areas												
Pallet Jack Wheels	NS	LW AT69	NS	NS	NS	NS	.	.	.	.	.	
Worker's Shoes	.	NS	NS	NS	NS	NS	.	.	.	.	.	
Entrance Door Handle-Outside	.	NS	NS	NS	NS	NS	.	.	.	.	.	
Room Entrance Door	.	NS	NS	NS	NS	NS	.	.	.	.	.	
In-Process Areas												
Screw Leg of 3660 (Cooking Device) Under Panel 6	NS	NS	.	.	.	.	.	.	.	.	.	
Chain Separating Pack and Cook	NS	NS	.	.	.	.	.	.	.	.	.	
Wall and Floor in Cook Room	NS	NS	.	.	.	.	.	.	.	.	.	
3660 Cooking Device-Panel 12	NS	NS	.	.	.	.	.	.	.	.	.	
Floor Cookside	.	.	NS	NS	NS	NS	.	.	.	.	.	
In-Process/Finished Areas												
Curve Belt Used To Transfer Product	.	.	.	.	.	.	.	.	.	.	.	
Transfer Bar Between Curve Belt and Individual Quick Freeze	.	NS	NS	.	NS	NS	.	.	.	.	.	
Wheel of Transfer Belt from Individual Quick Freeze to Packaging	NS	NS	.	LM DUP-1062A	.	.	.	.	.	.	.	

Squeegee in Packaging Room	NS	NS	LM DUP-1056A	LM DUP-1062D	.	.	LM DUP-1053A	.	.	.	.	
Individual Quick Freeze Belt	LW AT69	.	.	.	.	.	.	.	.	.	.	
Wheel of Blue Tote Cart in Packaging Room	NS	NS	.	.	.	.	.	.	.	.	.	
Weish Pack	NS	NS	.	.	.	.	.	.	.	.	.	
Blue Tubs	NS	NS	.	.	.	.	.	.	.	.	.	
Miscellaneous Table Packside	.	NS	NS	NS	.	.	.	.	.	.	.	
Employee Gloves	.	.	.	.	.	.	.	.	.	.	.	
Leg of Repack Table	NS	NS	.	.	.	.	.	.	.	.	.	
Curve of Conveyor Frame	NS	.	NS	.	.	.	.	.	.	.	.	
Wall on Packside	NS	NS	.	.	.	.	.	.	.	.	.	
Packside Drain	NS	.	.	LM DUP-1053A	.	.	LM DUP-1053A	.	.	.	.	
Transfer from 3660	.	.	.	.	.	.	.	.	.	.	.	

Cook side drain	NS	.	.	.	.	.	LM DUP-1052A	.	.	.	.	
Individual Quick Freeze Exhaust/Roof	NS	.	.	.	.	.	.	.	.	.	.	
Packaging Floor Exit	.	NS	NS	NS	NS	NS	.	.	.	.	.	
Koppens belt	.	.	NS	NS	NS	NS	.	.	.	.	.	
Floor Under Koppens Belt	NS	.	NS	NS	NS	NS	.	.	.	.	.	
Entrance Door Handle Inside	.	NS	NS	NS	NS	NS	.	.	.	.	.	
Packaging Room Floor	.	NS	.	.	.	.	.	.	.	.	.	
Individual Quick Freeze Exit Trap	NS	.	NS	NS	NS	NS	.	.	.	.	.	
Black Mats Packside Drain	.	.	.	LM DUP-1053A	.	.	.	.	.	.	.	
Incline conveyor	.	.	NS	NS	NS	NS	.	.	.	.	.	
Finished Product												
RTE Finished Prodcut	NS	NS	NS	NS	NS	NS	NS	.	.	.	.	

[a] NS indicates a site that was not sampled during a given sample collection and a "-" indicates that a sample did not test positive for *Listeria* during a given sample collection. *Listeria* positive samples are described by two capital letters to indicate the species that was isolated from the sample; where LM indicates *L. monocytogenes*, LI indicates *Listeria innocu*a, HLI indicates hemolytic *L. innocua*, and LW indicates *L. welshimeri*. *L. monocytogenes* molecular subtype is denoted (e.g., *Eco*RI ribotype DUP-1052A) and the *Listeria* spp. *sigB* allelic type (e.g., AT23) is denoted also denoted following species designation.

TABLE 3.8. *Listeria monocytogenes* and *Listeria spp.* contamination profiles for Plant 1 over a one-year period with bi-monthly sample collections.

Plant 6												
Sample Location	Dates Sampled											
	3/2007	3/2007	6/2007	7/2007	10/2007	12/2007	1/2008	3/2007	6/2007	7/2007	10/2007	12/2007
Raw/In-Process Areas												
Smokehouse Room Trough Floor Drain	LI AT70	LI AT31	LI AT70	.	LI AT70	.	.	LM DUP-1039C	LM DUP-1039C	LM DUP-1039C	.	.
Floor at Smokehouse Door	.	.	LI AT71	LI AT70	.	.	.	LI AT53	.	LI AT70	.	.
Floor Smokehouse Joint at South Smokehouse	.	.	.	.	.	LI AT53	LI AT53	LI AT53	LI AT53	LI AT70	.	LW AT43
Trench Floor Drain by South Smokehouse	LW AT16	.	LI AT70	LI AT23	.	LI AT53	.	LI AT53	.	LI AT53	.	.
Raw/In-Process/Finished Areas												
Smokehouse Truck Wheels	.	.	.	LI AT31	.	.	.	.	.	.	.	.
Packaging Room Squeegee	.	NS	NS	NS	NS	NS	.	.	.	.	.	.
Squeegee for Entire Plant Floors	NS	LI AT31	LI AT70	.	LI AT31	.	.	.	.	.	.	LI AT70
In-Process Areas												
Cooler Floor Drain by South Smokehouse	.	.	.	.	.	.	.	.	.	.	.	.
Cooler Door by South Smokehouse	.	.	.	.	.	.	.	.	.	.	.	.
Doors to Packaging Storage	.	.	.	.	.	.	.	.	.	.	.	.
Processed/Finished Areas												
Glove of Packaging Worker	.	NS	NS	NS	NS	NS	.	.	.	.	.	.
Cutting Board in Packaging Room	.	NS	NS	NS	NS	NS	.	.	.	.	.	.
Rallston Packaging Machine Loading Area	.	.	.	.	.	.	.	.	.	.	.	.

Metal Smokehouse Screen in Product Cooler	.	.	.	.	.	.	.	.	.	.	.	.
Plastic Smokehouse Screen in Product Cooler	.	.	.	.	NS	NS	.	.	.	.	.	.
Tabletop Under Cutting Board	.	.	.	.	.	.	.	.	.	.	.	.
Sliding Door Packaging to Meat Cooler	.	.	.	.	.	.	.	.	.	.	.	.
Trench Floor Drain in Packaging Room	.	LM DUP-1039C	LI AT70	.	.	.	.	LM DUP-1039C	.	.	LM DUP-1039C	.
Steel Table Leg by Floor Packager	.	LI AT70	.	.	.	.	.	LM DUP-1039C	.	.	.	.
Wheels to Slicer Cart in Packaging Area	.	LM DUP-1039C	LI AT70	NS	NS	NS	.	.	.	.	.	.
Slicer in Packaging Rooms	NS	.	NS	.	.	.	.	.	.	.	.	.
Table by Slicer	NS	.	.	.	.	.	.	.	.	.	LM DUP-1039C	.
Cooked Meat Cooler Floor Drain	LI AT70	LI AT31	NS	.	LI AT53	.	.	LM DUP-1039C	.	LI AT53	.	.
Frame of Metal Tree	NS	NS	NS	NS	.	.	.	.	.	.	.	.
Table Leg by Slicer	NS	NS	NS	LI AT70	.	.	.	.	.	.	.	.
Finished Product												
RTE Finished Product	NS	NS	NS	NS	NS	NS	.	.	.	.	.	LM DUP-1044A

[a] NS indicates a site that was not sampled during a given sample collection and a "-" indicates that a sample did not test positive for *Listeria* during a given sample collection. *Listeria* positive samples are described by two capital letters to indicate the species that was isolated from the sample; where LM indicates *L. monocytogenes*, LI indicates *Listeria innocu*a, HLI indicates hemolytic *L. innocua*, and LW indicates *L. welshimeri*. *L. monocytogenes* molecular subtype is denoted (e.g., *Eco*RI ribotype DUP-1052A) and the *Listeria* spp. *sigB* allelic type (e.g., AT23) is denoted also denoted following species designation

FIGURE 3.1. Dendrograms of all *Listeria monocytogenes* isolates collected from each of the six plants tested over a two-year period.

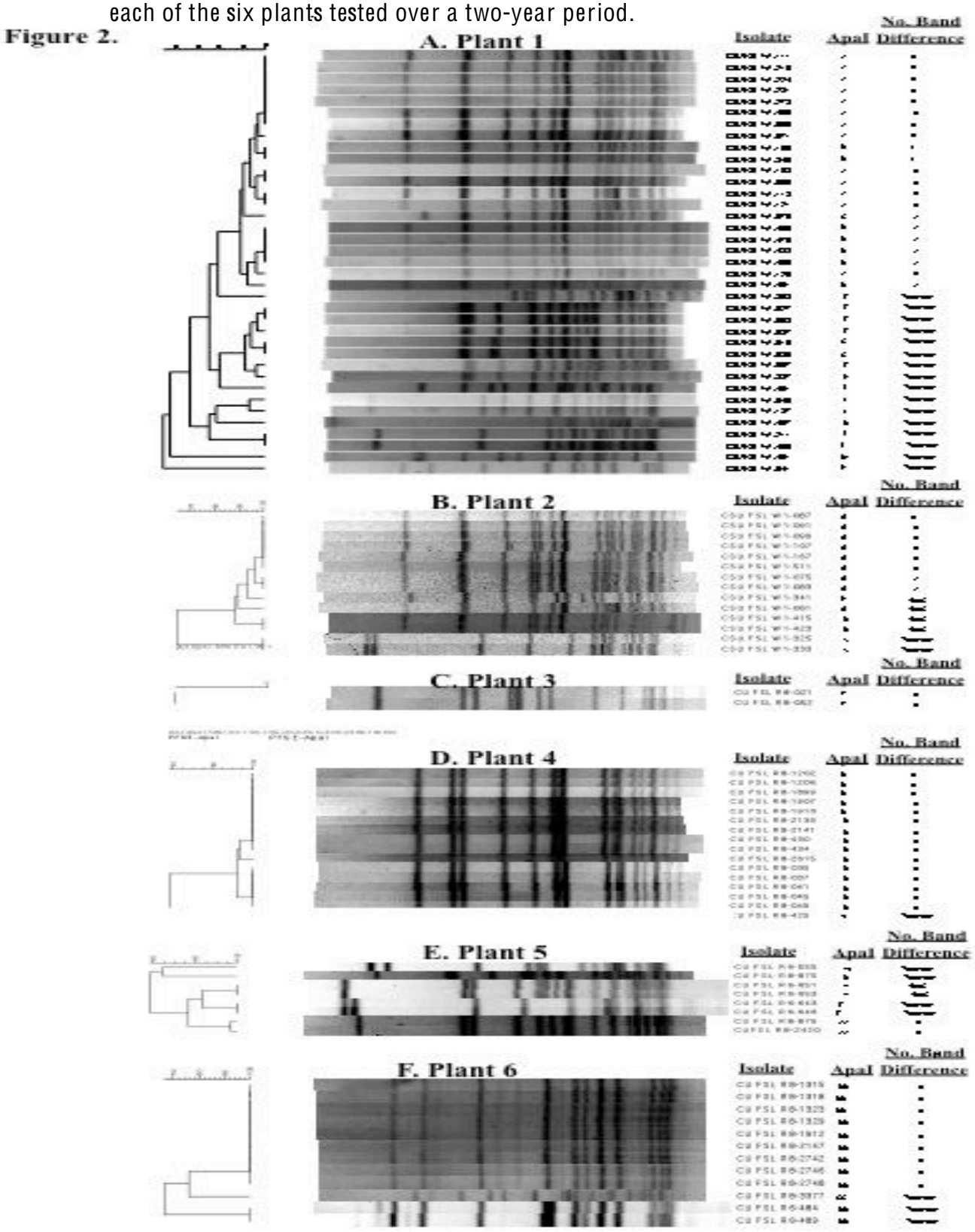

Figure 3.1. Dendrograms created using the unweighted pair group matching algorithm and the Dice correlation coefficient as implemented using BioNumerics software to visualize similarity of *L. monocytogenes* isolates from each RTE meat-processing plant. The first column following each PFGE patterns denotes the isolate identification (e.g., CSU FSL W1-041), the second column depicts the *Apa*I letter assignment given to that specific isolate (e.g., B) and the final column indicates the number of band differences between each isolate from the predominant PFGE subtype isolated from that specific plant. Dendrograms of *Apa*I digested DNA from *L. monocytogenes* isolates from plant 1-6 are depicted by Figs 1A-F, respectively.

FIGURE 3.2. Graph of pre- and post-test scores from in-plant training sessions given to employees at each of the six plants sampled.

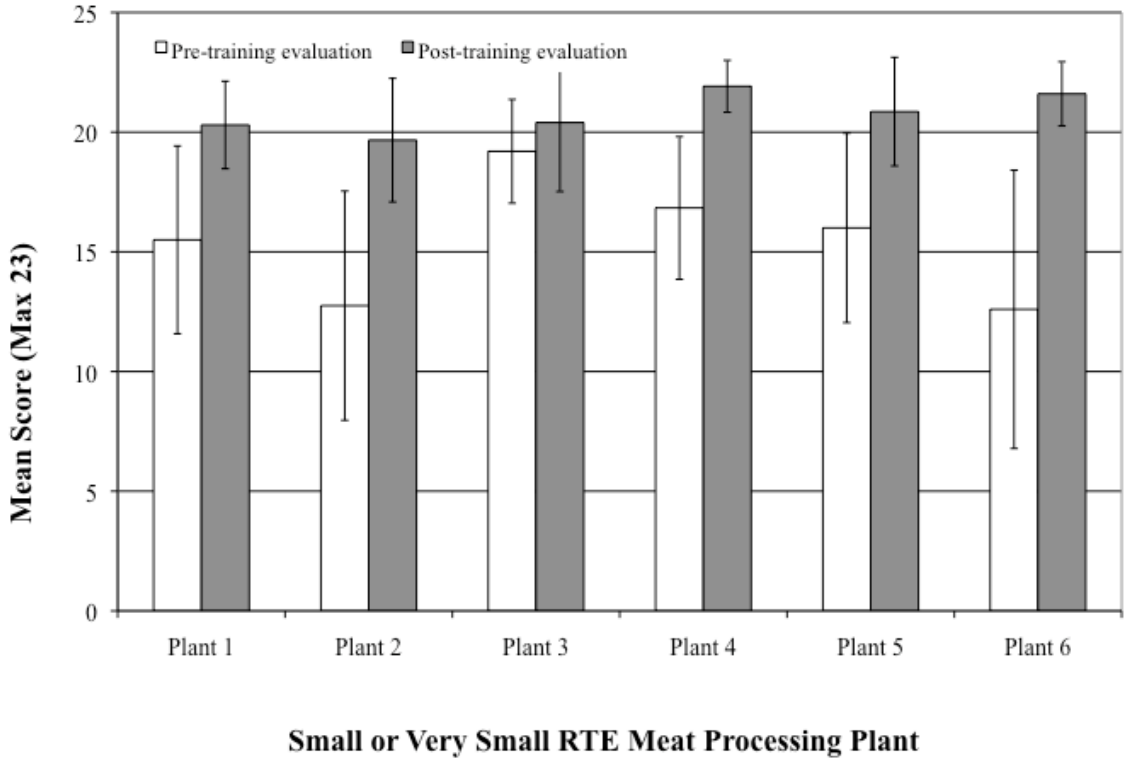

Figure 3.2. Pre- and post-training test scores for all employees across six small or very small RTE meat plants. The X-axis denotes each small or very small RTE meat plants enrolled in the study, including plants 1-6. The Y-axis indicates mean test scores before (open columns) and after (shaded columns) participation of all plant employees in an in-plant training session on *Listeria* ecology, listeriosis and control of *Listeria* in the RTE meat plant environment between year 1 and 2 of the project. Error bars denote standard deviation around the mean.