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DETECTING AND UNDERSTANDING THE PERSISTENCE OF A PREDOMINANT *LISTERIA MONOCYTOGENES* CLONE IN A COMMERCIAL FRESH MUSHROOM SLICING AND PACKAGING FACILITY

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ABSTRACT

A longitudinal survey of non-food-contact surfaces in a commercial mushroom slicing and packaging facility was conducted to determine the prevalence, distribution, and potential routes of transmission of *L. monocytogenes*. Samples were taken at 3 sampling occasions over a 13- month period. Multi-virulence-locus sequence typing (MVLST) was used to identify persistent and transient *L. monocytogenes* clones. The longitudinal study demonstrated that *L. monocytogenes* was present in 18.8% of samples. A trench drain and a wet porous concrete floor were identified as harborage sites for *L. monocytogenes*.

MVLST identified 4 different virulence types (VTs) of *L. monocytogenes* in the facility: VT11, VT107, VT105, and VT56. Of these clones, VT11 predominated and was persistent at 2 sampling sites during all sampling periods over 13 months. Improvements made in sanitation practices and facilities maintenance between the second and third sampling periods decreased the prevalence of *L. monocytogenes*; however, complete elimination of the pathogen was not achieved. Therefore, the persistent and transient clones were further evaluated for their ability to tolerate quaternary ammonium compound (QAC) sanitizer, adherence to stainless steel (SS) and concrete coupons, and growth rate and peak cell density in mushroom broth.

QAC tolerance among clones was investigated by growing them for 20-h (peak cell density) and 7 days (long-term-survival, (LTS), phase) at 35°C using minimum inhibitory concentration (MIC) and survival (tolerance) assays. The MIC value of QAC for all *L. monocytogenes* clones was 9.1 ppm. However, VT11 and VT107 grew to a significantly ($P \leq 0.05$) higher OD_{600} at sub-lethal QAC concentrations than VT105 and

VT56. In addition, LTS phase cells of VT11 were significantly ($P \leq 0.05$) more tolerant (2.6 to 220 times) to in-use QAC concentration of 200 ppm compared to other clones. Clones did not differ significantly ($P \leq 0.05$) in their ability to firmly adhere to concrete. However, on SS coupons, VT11 populations significantly ($P \leq 0.05$) decreased more rapidly than the other clones (after 5 and 7 days) suggesting the former dispersed earlier from the surface. Concrete coupons harbored more cells (2.12 to 8.06 log CFU/cm^2) than SS (1.14 to 6.12 log CFU/cm^2) and were significantly ($P \leq 0.05$) more protective of *L. monocytogenes* when treated with 200 ppm QAC.

Five different fresh mushroom broth (MB) dilution levels, 30%, 10%, 5%, 0.5% and 0.05%, were used to determine growth rate and peak cell density of *L. monocytogenes* clones at 35°C and 10°C. The results showed that all *L. monocytogenes* clones were able to grow and/or survive in each of the MB dilutions. The generation time of *L. monocytogenes* clones in MB ranged between 38.45 and 207.06 min at 35°C and from 9.27 to 28.42 h at 10°C. However, VT11 grew rapidly to a significantly ($P \leq 0.05$) higher peak cell density in 0.5% and 0.05% MB dilutions compared to other clones. The results indicate that VT11 may have a growth and survival advantage over other clones in nutrient-limited environments.

Overall, the results suggest that the observed predominance and persistence of VT11 in the surveyed mushroom slicing and packaging facility can be at least partially explained by 1) higher peak cell density at low mushroom nutrient levels, 2) higher growth at sub-lethal QAC concentrations, 3) higher tolerance to in-use QAC concentration in the LTS phase, and 4) a more rapid dispersal from surfaces allowing colonization of new areas in the mushroom processing environment. Based on these

results, a model was proposed to explain how several complex factors provide a competitive advantage for VT11 and thus the observed predominance and persistence within the mushroom processing facility. These results can be used to provide guidance on targeted interventions for reducing or controlling *L. monocytogenes* contamination in mushroom and other fresh produce processing facilities.

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List of Abbreviations

ANOVA	Analysis of Variance
ATP	Adenosine Triphosphate
CC	Clonal Complex
CDC	Centers for Disease Control and Prevention
CFIA	Canadian Food Inspection Agency
CFU	Colony Forming Units
DNA	Deoxyribonucleic Acid
EC	Epidemic Clone
EPS	Extracellular Polymeric Substances
FDA	Food and Drug Administration
FSIS	Food Safety and Inspection Service
LTS	Long-Term-Survival
MB	Mushroom Broth
MBC	Minimum Bactericidal Concentration
MIC	Minimum Inhibitory Concentration
MLEE	Multi-Locus Enzyme Electrophoresis
MLST	Multi-Locus Sequence Typing
MOX	Modified Oxford agar
MVLST	Multi-Virulence-Locus Sequence Typing
PBS	Phosphate Buffered Saline
PCD	Peak Cell Density

PFGE	Pulsed-Field Gel Electrophoresis
ppGpp	Guanosine PentaPhosphate
PPM	Parts Per Million
RPM	Revolutions Per Minute
RTE	Ready-To-Eat
SNP	Single Nucleotide Polymorphism
SS	Stainless Steel
ST	Sequence Type
TSAYE	Tryptic Soy Agar with 0.6% Yeast Extract
TSBYE	Tryptic Soy Broth with 0.6% Yeast Extract
QAC	Quaternary Ammonium Compound
USDA	United States Department of Agriculture
VT	Virulence Type

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Chapter 1

Statement of the problem

The demand for whole and fresh-cut produce has increased in recent years due to convenience and recognition of health benefits associated with consumption of fruits and vegetables. More recently, *Listeria monocytogenes* has become a growing problem for the fresh produce industry due to numerous outbreaks and recalls associated with consumption of ready-to-eat (RTE) fresh produce, including diced celery, stone fruits, caramel apples, mung-bean sprouts, and pre-cut salad.

In all these fresh produce associated outbreaks, *L. monocytogenes* was traced back to the processing environment in which the foods were packaged, indicating that the processing facility was the most likely source of *L. monocytogenes* contamination. However, to date, no studies have investigated the prevalence and persistence of *L. monocytogenes* in a commercial fresh-cut produce processing facility. Controlling or eliminating *L. monocytogenes* from RTE fresh-cut produce or processing facility is crucial because there is no *Listeria* kill step before consumption.

This is a major concern because *L. monocytogenes* is a dangerous foodborne pathogen with a mortality rate of ~30%. Elderly, immunocompromised, neonates, and pregnant women are at higher risk for listeriosis. Additionally, outbreaks and recalls caused by *L. monocytogenes* were reported to cause $2.8 billion economic loss in 2013. For these reasons, it is imperative to control or eliminate *L. monocytogenes* from RTE fresh-cut produce processing facility.

Fresh white button mushrooms (*Agaricus bisporus*) comprised 90% of the US mushroom market during 2014 – 2015. Of these, Pennsylvania accounts for 65% of the US mushroom production. The nutritional benefits of mushrooms have been recognized during the past decade resulting in increased mushroom consumption. Recalls of sliced mushrooms have recently occurred due to *L. monocytogenes* contamination and microbial surveys of commercially grown fresh produce have detected *Listeria* spp. on fresh mushrooms. Studies have shown that *L. monocytogenes* is capable of growing on whole and sliced *Agaricus* mushrooms, indicating that mushrooms provide an optimum growth environment for *L. monocytogenes*.

In addition, reflecting recent trends within the entire produce industry, mushroom growers and processors are facing increased scrutiny from wholesale buyers for evidence of proactive control measures to prevent pre- and post-harvest contamination of products with *L. monocytogenes* and other pathogens. Therefore, it is essential to determine the prevalence of *L. monocytogenes* in a mushroom processing facility and to identify high-risk areas to prevent *L. monocytogenes* contamination of mushrooms.

Previous surveys of RTE deli meats, dairy, pork, and seafood processing facilities reported that certain subtypes of *L. monocytogenes* predominate and persist in facilities for months to decades. Persistent subtypes of *L. monocytogenes* were defined as isolates that are repeatedly isolated on all sampling days and have similar phenotypic or genotypic characteristics. Subtypes that are not isolated on all sampling days or isolated in only certain sampling occasions have been termed transient. Persistent subtypes of *L. monocytogenes* have been reported to increase the risk of product contamination (*58, 180,*

203, *228*). Identification and elimination of these subtypes is necessary for effective control of *L. monocytogenes* within the facility.

Persistent subtypes have been isolated after routine cleaning and sanitizing (C&S) procedures. Therefore, differences in the ability of persistent and transient *L. monocytogenes* subtypes to grow in the presence of a sanitizer have been investigated by other researchers through minimum inhibitory concentration (MIC) assays. However, the results are inconclusive and moreover, these studies did not determine the ability of persistent and transient *L. monocytogenes* subtypes to survive (tolerate) bactericidal or in-use concentrations of sanitizer.

Previous studies have demonstrated that over time *L. monocytogenes* cells enter a non-replicating state with minimal metabolic activity termed the long-term-survival (LTS) phase (*94*, *255*). In other bacterial systems, this phase has been suggested to explain the persistence within an environment (*74*). Although most fresh produce processors rely on effective C&S procedures to eliminate *L. monocytogenes* from the facility, there is a lack of information regarding the effect of sanitizers on *L. monocytogenes* cells in the LTS phase.

Other widely investigated phenomena that may cause persistence of *L. monocytogenes* are adherence to surfaces and biofilm formation. Previous studies have used non-porous materials such as stainless steel, polyvinyl chloride microtiter plates, and glass slides for comparing the adherence and biofilm forming potential between persistent and transient subtypes of *L. monocytogenes*. Surveys from smoked salmon, cheese, and poultry processing facilities reported that porous surfaces such as floors and floor drains, made of concrete and cement, are major risk factors for *L. monocytogenes*

contamination (*9, 13, 73, 228, 230*). However, these porous surfaces were never tested to determine the differences in cell populations between persistent and transient subtypes.

Persistence of *L. monocytogenes* relies on growth and/or survival in a given environment. *L. monocytogenes* is always in competition for nutrients with other organisms in natural habitats and food processing environments; therefore, it is necessary for *L. monocytogenes* to utilize available nutrients in an effective manner for its growth and/or survival. Nevertheless, limited information is available regarding the differences in growth rate and survival in a food matrix from which the persistent and transient subtypes were originally isolated. This is important because differential utilization of nutrients in a competitive environment may offer a growth and/or survival advantage for persistent subtypes over transient subtypes.

Understanding the behavior of persistent and transient subtypes of *L. monocytogenes* is important because it could help explain why certain subtypes persist in certain locations in the food processing facilities. This information is critical for developing intervention strategies that eliminate or control *L. monocytogenes* contamination in mushroom and other food processing facilities, and thus can eliminate *L. monocytogenes* in RTE produce and other types of RTE foods.

Chapter 2

Literature review

2.1 Foodborne outbreaks and recalls

Outbreaks and recalls caused by foodborne pathogens significantly decrease public health and increase economic losses. In 2013, outbreaks and recalls contributed to an economic loss of $15.5 billion (*97*). Moreover, consumption of contaminated foods is estimated to cause 9.4 million foodborne illnesses every year with 55,961 hospitalized cases and 1,351 deaths (*187*). Nguyen et al. (*153*) showed that the number of foodborne outbreaks increased from 3,620 during 1973-1980 to 10,878 in 2001-2010. This increase in the number of outbreaks might be attributed to changes in agricultural practices, production, and distribution; and/or improvements in detection and surveillances techniques. Among these outbreaks, 230 were caused by known pathogens and occurred in multiple states in the United States. 47% of these outbreaks were caused by *Salmonella enterica*, 26% by shiga toxin-producing *Escherichia coli*, and 26% by *L. monocytogenes* (*153*).

2.1.1 Outbreaks attributed to contaminated fresh produce

The demand for fresh-cut fruits and vegetables has increased in recent years due to convenience and recognition of health benefits associated with the consumption of fresh fruits and vegetables. However, there are growing concerns in the fresh produce industry due to the increasing number of outbreaks and recalls caused by contamination with foodborne pathogens. Callejón et al. (*20*) reported that Norovirus (59%) is the major cause of outbreaks associated with fresh produce followed by *Salmonella* (19%), and

pathogenic *E. coli* (12%). *L. monocytogenes* accounts for < 1% of outbreaks associated with fresh produce, including sprouts in 2008 and diced celery in 2010 (*20*, *75*). However, the 2011 outbreak due to *L. monocytogenes* contamination of cantaloupes was one of the deadliest outbreaks in US history, causing 147 illnesses and 33 deaths (*75*, *140*). Since then, *L. monocytogenes* has caused multiple outbreaks involving novel vectors. For example, in 2014, *L. monocytogenes* caused outbreaks in stone fruits, caramel apples, and sprouts (*27*, *30*, *75*). In 2016, *L. monocytogenes* caused an outbreak in pre-cut salad (*28*). In all of these fresh produce outbreaks, *L. monocytogenes* was traced back to the processing environment in which the foods were packaged, indicating that the processing facility was the main source of contamination (*28*, *75*).

2.1.2 Contamination of fresh mushrooms with *L. monocytogenes*

L. monocytogenes has not caused outbreaks associated with the consumption of fresh commercially grown *Agaricus* mushrooms. However, microbial surveys of fresh produce have reported the presence of *Listeria* spp. on commercially grown fresh mushrooms (*91*, *185*). Additionally, several recalls of sliced mushrooms contaminated with *L. monocytogenes* have occurred recently (*31*, *32*, *33*, *34*, *212*). Studies have shown that *L. monocytogenes* is capable of growing on whole and sliced *Agaricus* mushrooms (*46*, *79*, *122*, *123*). However, sliced mushrooms have been shown to provide a more favorable environment for the growth of *L. monocytogenes* compared to whole mushrooms due to 1) the release of intracellular nutrients, 2) increased presence of water on sliced mushrooms, and/or 3) increased surface area for attachment and inoculation (*46*, *122*).

2.2 *Listeria*

2.2.1 Taxonomy of *Listeria*

The genus *Listeria* belongs to the division Firmicutes within the Family Listeriaceae. *Listeria* is closely related to other Gram-positive bacterial genera including *Bacillus, Clostridium, Staphylococcus,* and *Streptococcus. Listeria* are facultatively anaerobic, rod-shaped, non-spore-forming bacteria that were originally divided into six species: *L. monocytogenes*, *L. ivanovii*, *L. innocua*, *L. welshimeri*, *L. seeligeri*, and *L. grayi* (*218*). Other species of *Listeria* have recently been discovered including *L. weihenstephanensis, L. fleischmannii, L. floridensis, L. aquatica, L. cornellensis, L. riparia, L. grandensis*, *L. rocourtii,* and *L. marthii* (*52*, *81*, *186*).

2.2.2 *Listeria monocytogenes*

Among *Listeria* species, only *L. monocytogenes* is a food safety concern because it can cause the illness listeriosis in humans. The main mode of transmission of *L. monocytogenes* to humans is through the consumption of contaminated foods (*104*). Relative to other pathogens, the incidence of listeriosis in humans is low. Nevertheless, it has a hospitalization rate of 94% and is estimated to cause 255 deaths annually in the US (*187*). The high mortality rate (~30%) of *L. monocytogenes* makes it one of the deadliest foodborne pathogens. However, other species of *Listeria* including *L. ivanovii*, *L. innocua*, and *L. seeligeri* have been shown to sporadically cause illness in humans (*83*, *167*, *176*).

L. monocytogenes was first identified as an etiological agent in laboratory rabbits and guinea pigs in 1924 by EGD Murray, RA Webb, and MBR Swann (*150*). *L. monocytogenes* was considered an animal pathogen until 1981 when the first human *L. monocytogenes* outbreak associated with contaminated coleslaw caused 18 deaths and 41 illnesses (*95*). Since then, *L. monocytogenes* has been reported to be the cause of numerous foodborne outbreaks and recalls. It has been isolated from food production and processing facilities, smokehouses, slaughterhouses, soil, silage, water, and urban and natural environments (*12*, *68*, *78*, *182*, *196*, *222*, *233*). Therefore, effective control strategies to prevent *L. monocytogenes* contamination in foods and food supply systems are essential to increase public health and decrease economic losses associated with recalls and outbreaks.

2.2.3 Growth curve of *L. monocytogenes*

The growth curve of bacteria has traditionally been reported to consist of lag, log, stationary, and death phases. However, the existence of a fifth phase was originally observed in *Serratia* spp. by Steinhaus and Birkeland during 1939 (*194*). Lately, this fifth phase was also demonstrated in other bacteria such as *Escherichia coli* K12 (*67*), *Geobacter sulfurreducens* (*92*), and *L. monocytogenes* (*228*). In *L. monocytogenes*, this fifth phase is termed the long-term-survival (LTS) phase and historically, the cells in LTS phase were hypothesized to be in a dormant state (*228*). However, more recently, Gurresch et al. (*84*) reported that LTS phase cells of *L. monocytogenes* possess an intact cell membrane and high reductase activity suggesting evidence of metabolic activity; nevertheless, these cells showed no signs of replication.

The LTS phase cells of *L. monocytogenes* have been reported to transform back to actively growing log-phase cells during inoculation in fresh nutrients (*228*). The rate of formation of LTS cells depends on environmental conditions, initial cell density and life cycle stage (*54*, *230*). Cells of *L. monocytogenes* in the LTS phase have been reported to be more tolerant to high pressure processing and heat, compared to cells in other phases (*228*). The higher tolerance of LTS phase cells was speculated to be the result of cytoplasmic condensation, which further decreased the water activity within the cell and thereby reduced the rate of destruction by various processing methods (*228*).

Scanning electron micrographs of *L. monocytogenes* showed a transition from rod-shaped cells to coccoid cells during LTS phase (FIGURE 1) (*228*). In addition, the authors reported that several genes including *treB, glpF-2,* and *dnaK* were upregulated in the LTS phase (*229*). *TreB,* and *glpF-2* are involved in the transportation of compatible solutes such as glycine betaine and trehalose. Accumulation of compatible solutes within the cell has been shown to offer increased tolerance to cold and osmotic shock (*73*, *105*, *198*). *DnaK* acts as a molecular chaperone that offers protection against heat shock by preventing denaturation of essential proteins (*93*, *229*). *DnaK* has also been expressed in other stressful conditions including acidic pH and high salt concentrations, suggesting an overall protective effect against stresses (*93*). These changes in cell morphology and transcriptomic response might partially explain the increased tolerance of LTS phase cells under stressful conditions.

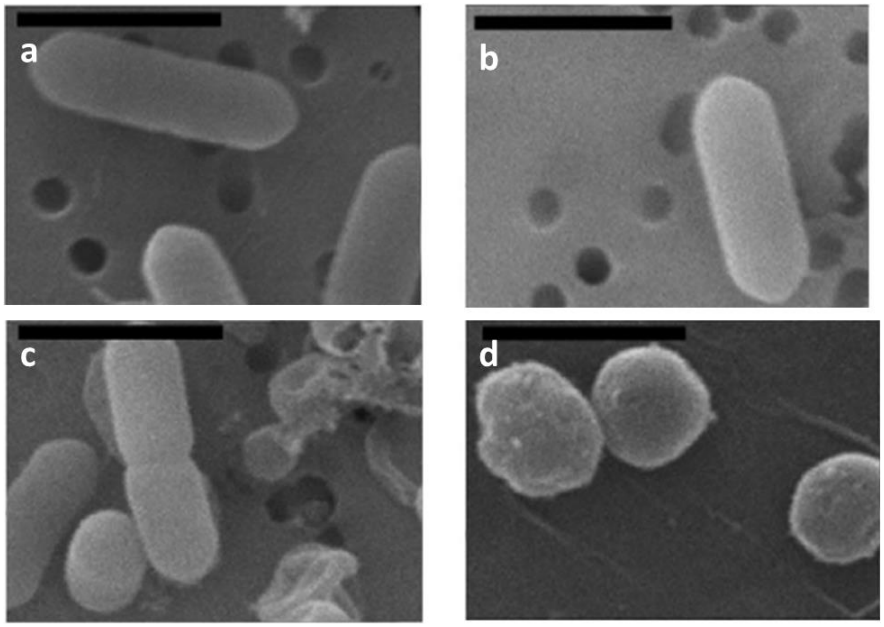

FIGURE 1: Scanning electron micrographs of *L. monocytogenes* at (a) late-log 12 h, (b) stationary 16 h, (c) late-death 41 h, and (d) long-term-survival 718 h phases when grown in tryptic soy broth supplemented with 0.6% yeast extract at 35°C.

Adapted from Wen et al. (*228*).

In addition to HPP and heat, LTS phase cells of *L. monocytogenes* were shown to be more tolerant to bactericidal concentrations of antibiotics, compared to cells in other phases (*54*). Antibiotic tolerant cells have classically been defined as persisters, which are a sub-population of cells that are tolerant to bactericidal concentrations of antibiotics (*110*, *124*, *232*). Recently, Doan et al. (*54*) showed that 100% of LTS phase *L. monocytogenes* cells were antibiotic tolerant and therefore, concluded that LTS phase cells can be classified as persisters. While the current definition of persister cells is limited to antibiotic tolerance, the term persister may need to be broadened to include tolerance to other types of stresses.

Persisters were first described by Joseph Bigger in 1944 who observed that a small sub-population of *Staphylococcus* spp. cells was not killed by the action of

penicillin (*14*). However, recent identification of persister cells in biofilms have generated more interest about the mechanisms involved in the formation of persisters. In other bacterial systems, the increased antibiotic tolerance of persister cells has been suggested to be the result of dormancy (*114*, *124*). Dormancy is induced by the production of the stress alarmone guanosine tetraphosphate (ppGpp), which results in activation of toxin-antitoxin systems, production of toxins and limited cell growth (*108*, *124*, *232*). However, in *L. monocytogenes*, these cells are clearly in a non-replicating metabolically state and limited information is available regarding the increased tolerance to antibiotics.

In conclusion, it is apparent that LTS phase cells of *L. monocytogenes* are phenotypic variants that are formed within a population as a survival mechanism and exhibit different cellular morphology, chemical composition and transcriptomic response compared to other life cycle phases. This cell variants are in a non-replicating metabolically active state and are more tolerant to processing conditions and antibiotics compared to other life phases. Nevertheless, the pathway(s) that leads to this state and the mechanism for increased stress tolerance are not clearly defined.

2.2.4 Growth characteristics of *L. monocytogenes*

L. monocytogenes cycles between saprophytic and pathogenic life styles (*72*). It is primarily found in soil and decaying vegetation (*72*). The optimum growth temperature for *L. monocytogenes* is between 30 and 37°C (*62*), water activity above 0.924 (*155*), and pH from 6 to 8 (*116*). However, it can survive under harsh environmental conditions, including high salt conditions (up to 40% w/v) (*127*), and a wide range of temperatures (-

0.4 to 45°C) (*62*), and pHs (3.0 to 12.0) (*127*). *L. monocytogenes* can utilize various carbohydrate molecules including glucose, glycerol, fructose, mannose, maltose, rhamnose, and chitin (*120, 207*). Sulfur containing amino acids such as cysteine and methionine were reported to be essential for growth of *L. monocytogenes* (*207*); however, valine, isoleucine, and leucine were required for growth in some strains of *L. monocytogenes* (*190*). Other micronutrients that promote growth of *L. monocytogenes* include vitamins such as biotin, lipoic acid, riboflavin, and thiamine, and amino acid supplements such as hemin and ferric citrate (*207*). *L. monocytogenes* is a catalase positive, oxidase-negative organism with a low guanine + cytosine content of 36 to 42% (*177*). Although *L. monocytogenes* lacks siderophores, it can hydrolyze esculin to sequester iron from the environment (*104*). It possesses peritrichous flagella and exhibits tumbling motility at temperatures below 30°C (*121*, *188*).

2.2.5 Pathogenesis of *L. monocytogenes*

L. monocytogenes is a strict intracellular pathogen that is capable of surviving and growing within mammalian host cells. The incubation period of *L. monocytogenes* in humans ranges from 3 to 70 days. The potential for acquiring listeriosis from contaminated foods depends primarily on dose level, immune status of the host, and virulence potential of the strain (*87*). The minimum infectious dose required to cause listeriosis is unknown, but studies have shown that cell populations as low as 100 to 1000 can cause illness in humans (*218*).

The immune response to a *L. monocytogenes* infection in humans is through cell-mediated immunity. Therefore, immunocompromised people, including pregnant women,

neonates, children, and elderly, with depressed T-cell-mediated immunity are much more susceptible to listeriosis than healthy populations (*104*, *218*). In immunocompromised people, *L. monocytogenes* invades epithelial cells in the gastrointestinal tract and enters the blood stream where it affects other parts of the body including spleen, liver, and brain (*169*). However, in healthy populations, *L. monocytogenes* can cause flu-like symptoms with few or no complications. Nevertheless, antibiotic treatments are recommended to prevent severe consequences of *L. monocytogenes* infection, such as septicemia, encephalitis, and meningitis.

The mechanism by which *L. monocytogenes* attacks mammalian epithelial cells is shown in FIGURE 2. Internalins such as *inlA* and *inlB* are surface proteins on *L. monocytogenes* that initiate its entry into host cells. *inlA* binds specifically to E-cadherin receptors on the host's epithelial cell surface. E-cadherin is an adhesion molecule that binds tissues together in the intestine, blood-brain barrier, and placenta (*169*). Conversely, *inlB* can bind to a wide range of host-cell receptors including hepatocyte growth factor, fibroblasts, and epithelioid cells (*104*, *126*). The binding of internalin is critical for infection because studies have reported that *L. monocytogenes* strains containing premature stop codons in internalins genes exhibited lower invasion efficiency compared to wild type strains (*65*, *154*).

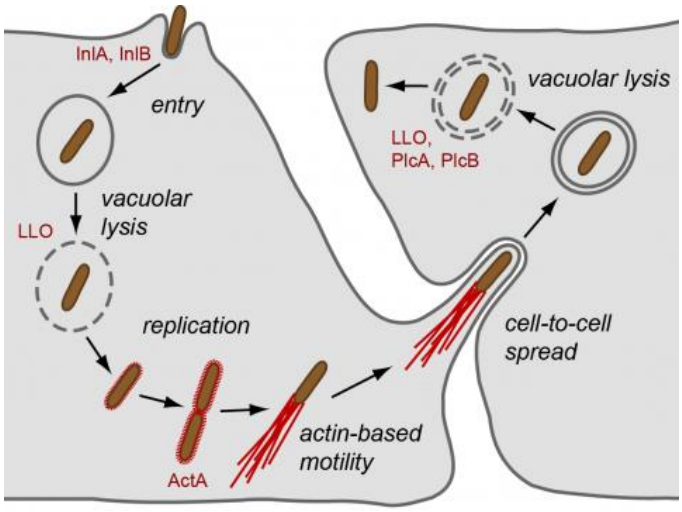

FIGURE 2: Intracellular spread of *Listeria monocytogenes* in mammalian epithelial cells.

Adapted from Pizarro-Cerda et al. (*169*)

Once *L. monocytogenes* cells adhere, they are internalized inside phagosomes. Listeriolysin O (*LLO*), a pore-forming toxin, is activated which causes the phagosomal membrane to lyse, thus releasing *L. monocytogenes* into the host cell cytoplasm where it multiplies. After replication, another surface protein (ActA) is synthesized which facilitates the formation of polarized actin tails that propel and transport *L. monocytogenes* from one host cell to another. This process of intracellular spread is called actin-based motility or F-actin polymerization (*146*, *169*). Once it enters adjacent cells, *L. monocytogenes* is phagocytosed in a double-membraned vacuole, which is subsequently lysed by the enzymes produced by *L. monocytogenes* such as *LLO*, phosphainositol phospholipase C (*plcA*), and phosphatidylcholine phospholipase C (*plcB*).

The above cycle is repeated causing the infection to spread throughout the surrounding tissues and to other organs. All of these virulence factors, *plcA, LLO, actA,*

and *plcB,* are encoded in a pathogenicity island cluster and regulated by the transcriptional activator *prfA* (*104*, *126*). *PrfA* expression is regulated by a RNA thermosensor mechanism that enables the translation of *prfA* proteins at 37°C (*72*). The expression of *prfA* is also controlled by the nutrients available in an environment. For instance, the presence of cellobiose signals *L. monocytogenes* that the environment is more suitable for a saprophytic life than a pathogenic life, which then downregulates *prfA* (*72*). This controlled expression of *prfA* and *prfA*-associated genes allow *L. monocytogenes* to balance efficiently switch between a saprophytic and an intracellular pathogenic life style.

2.2.6 Epidemiology

Epidemiology is the "study of the distribution and determinants of health-related states or events in specified populations, and the application of this study to the control of health problems" (*26*). During a *L. monocytogenes* outbreak or recall, the contamination source needs to be identified to stop the outbreak and thus minimize the number of illnesses. This requires expertise from both molecular and traditional epidemiologists who work together to pinpoint the source of contamination. Molecular epidemiologists identify the source of *L. monocytogenes* contamination using molecular biology techniques. After hypothesizing commonalities using these methods, traditional epidemiologic investigators conduct patient interviews or questionnaires to identify the source of the outbreak. Some terms that are widely used in molecular epidemiology include strain, clone, outbreak, and epidemic. A strain is defined as "isolates that have distinct phenotypic and/or genotypic characteristics from other isolates from the species"

(Chen and Knabel 2008). A clone is “a strain or group of strains descended asexually from a single ancestral cell that has identical or similar phenotypes or genotypes as identified by a specific strain method” (Chen and Knabel 2008). An outbreak is “an acute appearance of a cluster of an illness caused by an outbreak clone that occurs in numbers in excess of what is expected for that time and place” whereas an epidemic is “one or more outbreaks caused by an epidemic clone that survives and spreads over a long period of time” (Chen and Knabel 2008).

2.2.7 Subtyping methods

L. monocytogenes is categorized below the species level into subtypes. During an epidemiologic investigation of listeriosis, it is essential to detect these subtypes so that the contamination source(s) can be accurately identified. The most commonly used subtyping methods are described below.

2.2.7.1 Serotyping and serogrouping

L. monocytogenes was first characterized beyond the species level using serotyping. Serotyping is a phenotypic method that differentiates *L. monocytogenes* based on 15 somatic (O) and 5 flagellar (H) antigens (*104*, *161*). This alphanumeric system divides *L. monocytogenes* into 13 serotypes: 1/2a, 1/2b, 1/2c, 4a, 4b, 4c, 3a, 3b, 3c, 4d, 4e, 4ab, and 7 (*126*, *174*). Of these serotypes, 1/2a, 1/2b, and 4b account for 95% of human clinical cases, suggesting that these serotypes might possess some additional virulence factors that make them more virulent than other serotypes (*104*). However, due

to the low availability of antibodies, high cost, and inconsistent results, serotyping was later replaced by genoserogrouping.

Genoserogrouping uses polymerase chain reaction (PCR) to amplify the genes specific for certain groups of serotypes termed serogroups or genoserogroups (*58*). The advantages of this method are: high throughput, very small initial volumes, less time, and independent of the laboratory growth conditions. Using this method, *L. monocytogenes* was classified into 4 serogroups: I) 1/2a, 3a, 1/2c, 3c, II) 1/2c, 3c, III) 4b, 4d, 4e, 1/2b, 3b, and IV) 4b, 4d, 4e (*58*). The major disadvantage of this method is that it cannot differentiate serotypes within a serogroup.

2.2.7.2 DNA fragment-based methods

One of the most commonly used DNA fragment-based methods for subtyping is restriction fragment length polymorphism (RFLP). RFLP uses restriction enzymes to distinguish *L. monocytogenes* isolates based on differences in polymorphisms within and between DNA restriction sites. In this method, *L. monocytogenes* DNA is digested using restriction enzymes that can yield either short or large fragments. The digested DNA is separated by agarose gel electrophoresis into different-sized bands depending on their molecular weight. The isolates are then categorized based on their gel banding patterns. The most commonly used RFLP method is pulsed-field gel electrophoresis (PFGE) which uses rare cutting restriction enzymes like ApaI and AscI to digest DNA and characterizes *L. monocytogenes* isolates into different pulsotypes based on their banding patterns. PFGE is widely used in national laboratories for molecular subtyping; but it is rapidly being replaced by whole genome sequencing because PFGE is very tedious, time-

consuming, and labor intensive. Additionally, it cannot detect differences in nucleotide bases between strains and cannot determine phylogenetic relationships among isolates. Other fragment based methods include ribotyping (digest DNA using restriction enzymes and hybridize using a labelled rRNA probe) and randomly amplified polymorphic DNA (amplify DNA using short arbitrary primers for PCR)(*126*).

2.2.7.3 DNA sequence-based methods

DNA sequence-based subtyping methods discriminate *L. monocytogenes* subtypes at the single nucleotide level. DNA sequence-based subtyping methods detect differences in single nucleotides, termed single nucleotide polymorphisms (SNPs), from different subtypes and group them according to their presumed evolutionary relationship. Understanding evolutionary relationships between *L. monocytogenes* isolates is critical in an epidemiologic investigation for discriminating outbreak subtypes from non-outbreak subtypes and unrelated outbreak subtypes. DNA sequence-based subtyping methods include multi-locus sequence typing (MLST) (*171*), multi-virulence-locus sequence typing (MVLST) (*234*), whole-genome-sequencing (WGS) (*193*), and whole-genome multi-locus sequence typing (wgMLST) (*29*).

MLST groups *L. monocytogenes* isolates based on differences in 7 house-keeping genes and categorizes them as sequence types (ST) (*126*, *171*). The genes used in MLST analysis are *abcZ* (ABC transporter), *bglA* (beta glucosidase), *cat* (catalase), *dapE* (succinyl diaminopimelate desuccinylase), *dat* (D-amino acid aminotransferase), *ldh* (L-lactate dehydrogenase), and *lhkA* (histidine kinase) (*171*, *195*). MLST subtyping identifies clonal complexes (CCs) that are defined as “groups of clones that share 6 out of

7 allelic sequences with at least one other member of the group" (*22*). Currently, there are 641 STs and 22 defined CCs of *L. monocytogenes* (*99*, *171*, *195*). The most common CCs include CC1, CC2, CC5, CC6, CC7, CC8, and CC11 (*85*, *161*, *171*).

Alternatively, MVLST characterizes *L. monocytogenes* isolates based on differences in 6 virulence genes: *prfA* (listeriolysin positive regulatory protein), *inlB* (internalin B), *inlC* (internalin C), *dal* (alanine racemase), *clpP* (two-component response regulator), and *lisR* (clp protease proteolytic subunit) (*40*, *129*, *234*). MVLST categorizes *L. monocytogenes* isolates as virulence types (VTs) and to date, there are 138 VTs (*128*). MVLST has been shown to accurately differentiate epidemic clones, outbreak clones, and non-outbreak clones of *L. monocytogenes* with high discriminatory power and epidemiologic concordance (*41*, *109*, *234*). Additionally, MVLST has been used to identify contamination sources of *L. monocytogenes* in meat processing facilities (*137*) and a dairy herd (*86*). However, MVLST cannot differentiate multiple outbreak clones within a clonal group.

WGS is an emerging national surveillance tool to replace PFGE for tracking *L. monocytogenes* during epidemiologic investigations (*115*, *193*). The main advantage of this method is that it uses nucleotide sequences in the entire genome of *L. monocytogenes* rather than a few housekeeping (MLST) or virulence (MVLST) genes. This allows WGS to detect clusters of *L. monocytogenes* strains that are specific to a geographical location, thus allowing faster identification of contamination sources (*115*). It can also identify novel antibiotic resistance or virulence genes (*98*), phage genes (*3*), and plasmids (*23*). The most recently used method for identifying phylogenetic relationship is wgMLST

which is a derivative of WGS where thousands of loci are analyzed to determine differences in allelic variation between strains (*29*).

2.2.8 Lineages of *L. monocytogenes*

L. monocytogenes was first separated into 2 distinct phylogenetic groups by the multi-locus enzyme electrophoresis (MLEE) method (*168*), improvements in genetic characterization using virulence gene sequencing, DNA arrays, and ribotyping suggested 3 different phylogenetic lineages (*59, 172, 231*). Lineage I consists of serotypes 4b, 1/2b, 3b, 4d, and 4e whereas lineage II includes serotypes 1/2a, 1/2c, 3a, and 3c. Lineage III is composed of serotypes 4a and 4c with the exception of few strains from 4b serotype (*161, 172, 231*). Lineage 1 strains are predominantly found among clinical cases whereas lineage II strains are isolated largely from foods and food processing environments. Lineage III strains are primarily isolated from animal sources (*104, 161*). Differences in mortality have been observed between these lineages with lineage I serotype 4b causing 50 to 70% of human listeriosis cases (*104*). However, lineage II clones, serotype 1/2a, are now increasingly causing outbreaks and recalls (*109, 129*).

2.2.9 Epidemic clones of *L. monocytogenes*

Although *L. monocytogenes* consists of several clones, a subset of clones is known to cause epidemics, which can cause multiple outbreaks widely separated in time and space. An epidemic clone (EC) is further defined as “a strain or group of strains descended asexually from a single ancestral cell that is involved in one epidemic and can include several outbreak clones” (*39*). To date, seven ECs of *L. monocytogenes* have been

identified (*43*, *129*). ECI, ECII and ECIV consist of serotype 4b isolates, and ECIII and ECV consist of serotype 1/2a isolates. ECII, ECIII, and ECV are known to be specific for meat and poultry processing plants (*220*). A recent outbreak attributed to *L. monocytogenes* contaminated cantaloupe in 2011 led to the identification of 2 new ECs: ECVI and ECVII, which correspond to serotypes 1/2b and 1/2a, respectively (*129*). Some of these ECs, in particular ECIII and ECV, have been associated with human clinical cases and outbreaks for several years (*104*, *109*, *220*). These ECs have corresponding CCs through MLST subtyping as follows: ECI - CC1, ECIV - CC2, ECVI - CC5, ECII - CC6, ECVII - CC7, ECV - CC8, ECII - CC11 (*85*).

2.3 *L. monocytogenes* in foods and food processing facilities

2.3.1 Current regulations

In the United States, the presence of *L. monocytogenes* in a ready-to-eat (RTE) food product (25 g) or a food-contact surface is considered an adulterant and is subject to class I recall (*73*, *136*, *210*, *225*). In other words, a zero tolerance policy is now applied to the presence of *L. monocytogenes* in RTE food products in the US. When a food-contact surface and/or food that can support the growth of *L. monocytogenes* is tested positive for *L. monocytogenes*, the mandated corrective actions are to recall all food products produced through the contaminated food-contact surface, destroy the contaminated food, reprocess the food to kill *L. monocytogenes*, or divert the food to animal use (*210*, *211*). When *Listeria* spp. is detected on a food-contact surface, recommended corrective actions include testing product for the presence of *L. monocytogenes*, conducting additional sampling, improving sanitation standard operating procedures, follow up

sampling, identification of the cause, implementing and documenting corrective actions (*136*, *210*, *211*).

2.3.2 Prevalence of *L. monocytogenes*

Because *L. monocytogenes* is a ubiquitous organism, contamination of foods with *L. monocytogenes* can occur at any stage of the food supply chain including production farms, processing environments, and retail stores (*12*, *68*, *78*, *182*, *191*, *196*, *222*, *233*). The main factors that affect the prevalence rate of *L. monocytogenes* at these stages are the presence of food, water, time, temperature, and efficacy of cleaning and sanitizing procedures (*6*, *133*, *205*). In addition, the prevalence of *L. monocytogenes* was also affected by seasons in dairy farms (*77*).

In studies by Strawn et al. (*196*) and Weller et al. (*227*), *L. monocytogenes* was isolated from 17.5% of produce fields, 39% of non-irrigation water, and 9% of irrigation water samples. Silage and ruminant animals were reported as sources of *L. monocytogenes* (*64*, *69*). *L. monocytogenes* has been detected from 93.3% and 6.7% of samples taken from urban and natural environments, respectively (*227*). Although elimination of *L. monocytogenes* from the farm environment is impossible, a recent study by Weller et al. (*227*) suggested that measures taken at the irrigation level can help reduce the prevalence of *L. monocytogenes* in the produce farm environment.

Post-process contamination of foods by *L. monocytogenes* is a major concern in the food industry. Routes of transmission of *L. monocytogenes* to finished products in the processing facility can occur through incoming raw materials, pests, processing aids such as brine solutions, compressed air, ice, food and non-food-contact surfaces such as

equipment, employees, floors, floor drains, and water condensate (*12*, *133*, *144*, *199*, *205*, *211*). Wet or moist and cold areas in the packing houses and processing environments have repeatedly been reported to be sources of *L. monocytogenes* contamination (*5*, *66*, *75*, *94*, *133*, *203*). TABLE 1 shows the prevalence of *L. monocytogenes* isolated from various processing facilities.

TABLE 1: Overall prevalence of *L. monocytogenes* in food processing facilities including raw materials, food-contact and non-food-contact surfaces, and finished products.

Processing facility	***Listeria* spp.**	***L. monocytogenes***	**Reference**
Cheese	21.9%	19.5%	(*184*)
Farmstead Dairy	14.8%	3.4%	(*94*)
Milk	19.52%	7.62%	(*55*)
Mussel	NA	1.12%	(*49*)
Smoked Fish	20.7%	8.2%	(*203*)
Cold-smoked salmon	NA	42%	(*51*)
Crawfish	20.6%	3.2%	(*118*)
RTE deli meats	14.8%	9.5%	(*191*)
Dried Sausage	NA	31%	(*202*)
Beef	75.5%	18.7%	(*175*)
Produce (Frozen)	63%	41.3%	(*166*)

NA- not available. Prevalence of generic *Listeria* spp. was not determined in the study.

2.3.3 Growth and survival of *L. monocytogenes* under different environmental conditions in food facilities

2.3.3.1 Cold

Produce processing facilities generally operate at cold temperatures to maintain produce quality. *L. monocytogenes* is a psychrotrophic organism and can therefore grow at refrigerated temperatures (40°F). At low temperatures, bacterial cell membranes typically become rigid causing reduced transport of molecules in and out of the cell. However, *L. monocytogenes* overcomes the membrane rigidity by modifying the

composition of fatty acids in the cell wall. These modifications include an increase in the ratio of C15:0 to C17:0 fatty acids, increased production of unsaturated fatty acids, and an increase in the ratio of ante-iso to iso-fatty acids (*73*, *201*). These changes decrease interactions between adjacent carbon molecules, thus increasing membrane fluidity at low temperatures (*73*). Other factors involved are the accumulation of compatible solutes such as glycine betaine and carnitine, changes in gene expression, production of cold shock proteins (Csps), and activation and upregulation of the universal stress sigma factor sigB *(σ^B)* (*73*, *151*, *201*). Such changes are required to maintain enzyme activity and transport of solutes across the membrane.

2.3.3.2 Acid and salt

Acidification and salt addition are used as control measures to prevent growth and survival of pathogens in foods. However, *L. monocytogenes* grows at a wide range of pHs (5 to 11) (*127*) and salt conditions (0 to 10%) (*174*), thus presenting more challenges for eliminating the pathogen in acidic and salty foods. Under acidic conditions, *L. monocytogenes* cells maintain intracellular pH by utilizing several regulatory systems, including glutamate decarboxylase, σ^B, and *lisRK* (*42*, *73*). When exposed to high salt concentrations, *L. monocytogenes* maintains turgor pressure by taking up compatible solutes and by regulating the gene expression of salt shock proteins (*19*, *73*, *74*).

2.3.3.3 Surface adherence and biofilm formation

Generally, bacteria exist in two different states: planktonic or attached to surfaces. *L. monocytogenes* has been shown to attach to surface materials widely used in food

facilities. These include stainless steel (SS), polyvinyl chloride, buna-N-rubber, high density polyethylene, polyester floor sealant, glass, and food-conditioning-films (FCFs) (*11*, *15*, *53*, *220*). Once attached, *L. monocytogenes* can form biofilms on surfaces (*35*, *160*).

Simple and elaborate definitions of biofilm used by researchers include "a biofilm is a community of microbes associated with a surface, typically encased in an extracellular matrix" (*143*), and "biofilm is a microbially derived, sessile community characterized by cells that are irreversibly attached to a substratum or interface, or to each other; are embedded in a matrix of extracellular polymeric substances that they have produced; and exhibit an altered phenotype, with respect to growth rate and gene transcription" (*56*).

Attachment and biofilm formation involve five major steps: 1) reversible attachment, 2) irreversible attachment, 3) multiplication, 4) production of extracellular polymeric substances (EPS), and 5) dispersal. The EPS layer of *L. monocytogenes* consists of extracellular proteins, polysaccharides, and DNA (*47*, *88*, *152*). These stages of attachment and biofilm formation have been summarized by Garrett et al. (*76*) and Van Houdt and Michiels (*216*). The degree of attachment and biofilm formation depends on the 1) presence of surface appendages (*121*), 2) property of the surface material (*36*, *178*), 3) effect of food residues (*170*, *220*), 4) influence of other microbial populations (*18*, *236*), and/or 5) environmental factors such as temperature, salt, and pH (*159*, *216*).

Several different methods were used to determine attachment and biofilm formation by *L. monocytogenes*, including 1) microtiter plate assays using crystal violet (*16*, *53*, *224*), 2) culture slide assays using ruthenium red or acridine orange (*164*), 3)

growing *L. monocytogenes* on different surface materials and dislodging the cells using sonication, swabbing, or vortexing using glass beads (*10*, *18*, *170*), and 4) FCFs (*220*).

The microtiter, culture slides, and FCF assays are rapid methods with a very high throughput. However, these methods can overestimate the amount of biofilm formed due to the stain binding to carbohydrates present on both dead and viable bacterial cells, the EPS matrix, as well as metabolites produced by bacteria (*15*). The plating method can determine the ability of *L. monocytogenes* to attach and form biofilms on different surface materials as well as allows enumeration of viable cells. However, it is time consuming and it cannot characterize the biofilm architecture or the amount of EPS layer produced by *L. monocytogenes*.

Using the above mentioned methods, studies have found some relationship between lineages and biofilm formation (*89*, *157*, *215*, *220*). Some studies demonstrated that lineage II strains form greater amounts of biofilms compared to lineage I strains in nutrient-limited conditions on SS (*89*, *157*, *214*), whereas lineage I strains formed dense biofilms on SS and microtiter plates under high nutrient conditions (*53*, *215*). Verghese et al. (*220*) showed that the type of FCFs can affect the growth and biofilm formation of ECs of *L. monocytogenes*. The authors reported that ECs isolated from meat processing facilities formed denser biofilms on meat-conditioning films compared to other FCFs (*220*). They concluded that these ECs may have adapted to the food matrix from which they were originally isolated, resulting in increased growth and biofilm formation (*220*). In general, growth media used in these studies seems to have a major impact on the number of cells attached to a surface.

2.3.3.4 Antimicrobial compounds

Antimicrobial compounds, organic acids, sanitizers, antibiotics, and bacteriocins, are used for controlling the growth and survival of foodborne pathogens. Some *L. monocytogenes* subtypes have been reported to be resistant to sanitizers and some class of antibiotics (*60*, *80*, *112*, *141*, *148*, *192*). The terms "resistance" and "tolerance" were used interchangeably in several studies (*60*, *90*, *224*); however, this might sometimes lead to false interpretation of results including underestimation of the sanitizer efficiency and overestimation of tolerance by bacterial strains (*60*, *224*). Consequently, in our study we define resistance to an anti-microbial compound as the ability of a bacterial strain to grow at concentrations that are bactericidal for other strains within a species and tolerance is the ability of a strain to survive temporary exposure to concentrations above the MIC (*17*).

This section is focused on the effect of sanitizers used in the food industry on *L. monocytogenes* cells. This is a major concern for food processors including fresh produce processors who rely on cleaning and sanitizing practices to eliminate *L. monocytogenes* from the processing environment, thus preventing post-process contamination of RTE products. Active ingredients in most commonly used sanitizers include quaternary ammonium compounds (QACs), sodium hypochlorite, and peroxyacetic acid (*48*, *144*). The effectiveness of a sanitizer depends on composition, concentration, contact time, influence of organic matter, temperature, pH, water hardness, and the physiological state of bacteria (planktonic versus attached) (*48*, *165*, *179*).

2.3.3.5 Quaternary Ammonium Compounds (QACs)

Quaternary ammonium compounds (QACs) are a class of cationic sanitizers commonly used in food facilities for sanitizing purposes. QAC were reported to be effective against Gram-positive bacteria compared to Gram-negative bacteria (*130*). At low concentrations, QACs bind to negatively charged groups on the bacterial cell wall (e.g. proteins) where they disrupt membrane permeability and cause cytosolic leakage. At higher concentrations, QACs also penetrate into the cell where they target carboxylic groups and cause cytoplasmic coagulation (*204*).

According to FDA regulations, the recommended "in-use" no rinse concentration of active QAC for food-contact surface sanitizing is 200 ppm (*213*). But the concentrations used on non-food-contact surfaces can vary between type of surfaces (porous vs non-porous), manufacturer, and chemical composition of sanitizer. The minimum inhibitory concentration (MIC) values of QAC for *L. monocytogenes* range from 0.63 to 32 ppm, while the growth of most *L. monocytogenes* subtypes is inhibited at concentrations below 4 ppm (*1*, *132*, *141*, *204*) and the minimum bactericidal concentration (MBC) of QAC was 35 ppm (*63*). However, some studies have shown that during short-term exposure, *L. monocytogenes* can adapt to sub-lethal QAC concentrations which can lead to increased tolerance at higher concentrations (*132*, *204*). Nevertheless, when applied at the manufacturer's recommended concentration, the concentration is sufficient to kill *L. monocytogenes* on areas that are adequately cleaned and sanitized.

On the other hand, a major concern in the food industry is the high tolerance of biofilms to sanitizers (*48*, *144*, *164*). Studies have shown that surface-attached *L. monocytogenes* cells or biofilms are 31 to 2500 times more tolerant to QAC than their planktonic counterparts (*48*, *113*, *152*). This increased sanitizer tolerance by biofilms is due to 1) insufficient sanitizer contact due to low diffusion through the EPS layer, 2) changes in cell physiology owing to the slower growth rate, and/or 3) differential gene expression in biofilms compared to planktonic cells, including down regulation of flagellin proteins and upregulation of stress-induced proteins (*125*, *144*, *152*, *206*).

2.3.4 Persistence of *L.* monocytogenes subtypes in food facilities

Certain subtypes of *L. monocytogenes* have been shown to preferentially predominate and persist in food facilities (*69*, *86*, *106*, *137*, *145*, *162*). Persistence of *L. monocytogenes* has been defined as "the repeated isolation of *L. monocytogenes* strain(s) on different sampling days that has/have identical subtypes which is determined either by phenotypic or genotypic methods" (*66*). The ability of *L. monocytogenes* to colonize food processing facilities was first documented by Rørvik et al. (*182*). The authors showed that an electrophoretic type - 6 categorized by MLEE persisted in a cold-smoked salmon processing facility for 8 months. Since then, studies have reported persistence of specific subtypes for months to decades in other facilities, including mussels (*49*), cold-smoked salmon (*51*), cheese (*5*, *184*), crawfish (*118*), chicken (*12*), RTE meals (*106*), and bulk milk tanks (*149*). Persistent subtypes of *L. monocytogenes* have been repeatedly isolated from the environment and have caused recurrent episodes of finished product contamination.

2.3.4.1 Raw materials

Routes of transmission of *L. monocytogenes* to finished products can occur either through incoming raw materials or the processing environment including equipment, employees, floor, and drains. A study by Berrang et al. (*12*) showed that a chicken processing plant was colonized by *L. monocytogenes* through incoming raw chicken. Studies from smoked-fish and crawfish facilities have also demonstrated that raw materials are the source of persistent *L. monocytogenes* contamination in the processing environment as well as in finished products (*61*, *118*, *156*).

2.3.4.2 Processing environment

Besides raw materials, the processing environment is also a major source of persistent *L. monocytogenes* contamination (*51*, *162*, *182*, *203*). Thimothe et al. (*203*) reported that *L. monocytogenes* subtypes isolated from finished products grouped with subtypes from the processing environment rather than the raw materials, suggesting that the processing environment was the source of persistent subtypes within the smoked-fish processing facility. Other studies have also shown that the risk of product contamination increases when the processing environment becomes contaminated with *L. monocytogenes* (*96*, *106*, *205*, *233*).

Persistent subtypes of *L. monocytogenes* have been most frequently isolated from floors, drains, slicers, and dicers (*8*, *12*, *82*, *102*, *203*). Due to their complex design, these areas are often not adequately cleaned and sanitized, and thus can act as harborage sites for *L. monocytogenes.* A harborage site has been defined as a “set of biotic and abiotic

conditions in which a species is able to persist and maintain stable population sizes" (*214*). *L. monocytogenes* contamination can also spread through gloves and aprons of employees, and job rotation between departments (*7*, *21*, *181*).

2.3.5 Factors causing persistence of *L. monocytogenes* subtypes in food processing facilities

Previous studies have suggested that persistence of *L. monocytogenes* subtypes within a facility is due to either random (stochastic) events and/or adaptation to the environmental conditions in the facility (*66*). However, there is not sufficient information to demonstrate that stochastic events contribute to persistence of *L. monocytogenes* in the processing facilities. Conversely, few studies reported that certain subtypes can quickly adapt to the processing conditions resulting in improved adherence to surfaces and biofilm formation (*135*, *220*, *224*), higher tolerance to sanitizers and heavy metals (*148*), and resistance to phage attack (*107*). Some of the studies that have determined the differences between persistent and transient subtypes in certain characteristics are described below.

2.3.5.1 Adherence and biofilm formation

Persistent subtypes of *L. monocytogenes* were found to adhere 2- to 11- times higher on SS after 1- and 2- h contact times, compared to transient subtypes (*135*). Nevertheless, the differences in adherence between persistent and transient subtypes diminished after 72 h. Moreover, in some instances, the adherence of transient subtypes on SS was greater than persistent subtypes (*135*). Studies conducted on microtiter plate

assays have also demonstrated increased adherence by persistent subtypes compared to transient subtypes (*119*, *191*, *224*). In contrast, some studies have reported no significant differences in adherence and biofilm formation between persistent and transient *L. monocytogenes* subtypes (*53*, *89*).

All these studies have focused on non-porous SS and microtiter plates to determine the efficiency of adherence between subtypes; however, porous surfaces such as concrete and cement that were reported as major risk factors of *L. monocytogenes* contamination (*9*, *13*, *73*, *228*, *230*) were never evaluated for differences in cell populations between persistent and transient subtypes.

2.3.5.2 Sanitizer tolerance

Persistent subtypes of *L. monocytogenes* have been reported to possess increased tolerance to QAC compared to transient subtypes. A study by Fox et al. (*70*) revealed 92 genes had differential expression when persistent and transient subtypes were exposed to 4.0 ppm QAC. Of these, genes involved in peptidoglycan synthesis were 2.08 to 35.16 times upregulated in persistent subtypes compared to transient subtypes. A study by Casey et al. (*25*) demonstrated upregulation of 600 genes when a persistent subtype isolated from a cheese farmhouse was grown in the presence of 4.0 ppm QAC, compared to 0 ppm QAC. However, this study did not test the transcriptomic response of non-persistent subtypes. Other studies have also shown that the presence of efflux pumps, *bcrABC* cassette, upregulation of certain set of genes can contribute to persistence of certain subtypes (*2*, *60*, *180*, *200*). However, other authors did not find any correlation between persistence and tolerance to QAC (*90*, *103*, *131*, *224*).

Some of these studies used an overnight-grown culture and one sub-lethal concentration of sanitizer (QAC) to test the hypothesis that persistent subtypes are more tolerant to sanitizers than transient subtypes. The use of a sub-lethal concentration might be representative for harborage sites where cleaning and sanitizing might be inadequate. However, persistence involves long-term survival in these harborage sites. At these sites, *L. monocytogenes* might be present in different phases of life cycle, including LTS phase. Nevertheless, the response to sanitizer by LTS phase cells has not yet been investigated.

2.3.5.3 Native microflora

L. monocytogenes live in communities with other microorganisms. Therefore, it is imperative to understand the influence of native microflora on clonal selection of *L. monocytogenes.* A study by Zhao et al. (*235*) demonstrated that *Enterococcus durans, Lactococcus lactis* subsp. lactis, and *Lactobacillus plantarum* can inhibit planktonic and SS adhered *L. monocytogenes* cells. On the other hand, Schirmer et al. (*189*) showed that the native microflora did not inhibit the growth of *L. monocytogenes;* however, persistent and transient subtypes were not used in this study. Other studies have shown that *Flavobacterium*, *Rhodococcus*, and *Pseudomonas* have a positive effect on adherence of *L. monocytogenes* whereas *Bacillus*, *Janthinobacterium*, and *Staphylococcus* inhibited the adherence of *L. monocytogenes* to surfaces (*18*, *71*, *100*, *237*). However, these studies only focused on the influence of the native microflora on *L. monocytogenes* attachment to surfaces. The influence of the food matrix and native flora on growth and survival of *L. monocytogenes* subtypes have not yet been studied.

2.3.5.4 Genetic determinants

Genotypic elements of *L. monocytogenes* that may affect the potential for persistence in food processing facilities have been reported in several studies. Subtypes containing *comK* prophage junction fragments adhered more to FCFs and formed denser biofilms than subtypes lacking the *comK* prophage region (*220*). Studies conducted by Mullapudi et al. (*147*) and Ratani et al. (*173*) on *L. monocytogenes* isolates obtained from several processing facilities showed that subtypes with cadmium resistant plasmids were more tolerant to benzalkonium chloride suggesting that a competitive advantage over other subtypes that are devoid of plasmids with heavy-metal resistance. Another biological element that may affect persistence of certain *L. monocytogenes* subtypes is bacteriophages. Studies from turkey and smoked fish processing facilities showed that certain subtypes of *L. monocytogenes* were resistant to certain types of phages suggesting that this phage-resistant mechanism can also confer preferential persistence of specific subtypes over others (*107*, *223*).

Overall, majority of these studies described that persistent subtypes contain unique features compared to transient subtypes. On the other hand, Tompkin (2002) and Carpentier and Cerf (2011) suggested that persistent subtypes do not possess any unique characteristics compared to transient subtypes because of the discrepancy in results between studies. However, we speculate that persistence of *L. monocytogenes* within a food facility does not primarily depend on a single characteristic of the organism, but might involve complex interactions with biotic and abiotic factors present within the environment.

Despite differences between studies, persistent strains are often isolated from areas that are difficult to clean and sanitize. These areas, however, can act as harborages sites for *L. monocytogenes* and it is more likely that adaptation to these sites may provide a competitive advantage for specific subtypes and cause persistence. It is also possible that a slight increased rate of adaptation (increased adherence, higher sanitizer tolerance, and/or other characteristics) by persistent subtypes might become significant over time and yield a competitive advantage over transient subtypes. Therefore, an improved understanding of whether and/or how persistent subtypes undergo niche adaptation would help to understand the clonal selection of *L. monocytogenes* and development of targeted intervention strategies for controlling and eliminating persistent *L. monocytogenes* contamination in food facilities. However, persistence due to stochastic events cannot be ignored.

2.3.6 Control of *L.* monocytogenes in food processing facilities

Because *L. monocytogenes* is a ubiquitous organism, its introduction into production and/or processing facilities through raw materials, employees, equipment, and farm animals is inevitable. However, effective cleaning and sanitizing practices have been shown to reduce *L. monocytogenes* contamination in food facilities (*117*, *118*). Malley et al. (*136*) suggested that a "seek and destroy" strategy can help decrease the prevalence of *L. monocytogenes* within a facility. This process involves identification of areas with recurrent contamination or harborage sites so that effective targeted approaches can be implemented. This is in agreement with other studies where targeted

intervention measures were shown to reduce or eliminate *L. monocytogenes* from facilities (*106*, *117*, *118*, *133*, *197*).

Other control strategies suggested by researchers to control the colonization and spread of *L. monocytogenes* include 1) equipment sanitary design improvements that facilitate effective cleaning and sanitizing, 2) *Listeria* environmental monitoring program, 3) periodic rotation of sanitizers, 4) improvements in facility design and maintenance so they are free from structural defects such as floor cracks and crevices, and 5) compartmentalization and restriction of traffic flow between raw and finished product areas (*50*, *134*, *136*, *141*, *144*, *205*).

Floors and floor drains were identified as sources of *L. monocytogenes* contamination in several processing facilities. Tompkin (2002) suggested that scrubbing floors and floor drains with caustic soda followed by rinsing, application of sanitizer, and air drying can control *L. monocytogenes* contamination. Jessen and Lammert (2003) also suggested that scrubbing was the most effective cleaning method to eliminate *L. monocytogenes* from floors. However, possible damages caused by these treatments must be taken into consideration when implementing these techniques. A study by Paiva et al. (*163*) showed that the use of a concrete sealant before and after *L. monocytogenes* inoculation was effective in reducing *L. monocytogenes* counts.

A major concern for the use of sanitizers to kill *L. monocytogenes* is the development of resistance and/or tolerance to sanitizers over time. To prevent this, studies have recommended periodic rotation of sanitizers with different modes of action (*101*, *141*). Conversely, a study by Lundén et al. (*132*) showed that *L. monocytogenes* exposed to sub-lethal concentrations of a sanitizer cross-adapted it to both chemically

related and unrelated sanitizers. This brings to question whether sanitizer rotation is effective for preventing development of resistance and/or tolerance and suggests that multiple strategies are necessary to effectively control persistent *L. monocytogenes* contamination in food facilities.

Detection of *L. monocytogenes* on a food-contact surface triggers a mandatory recall of all products processed through the contaminated food-contact surface. Therefore, in their environmental testing programs, food processors often test for *Listeria* spp. instead of *L. monocytogenes.* However, the use of *Listeria* spp. as an indicator for *L. monocytogenes* has been demonstrated to be ineffective in produce fields (*37*) and seafood processing facilities (*4*). Alali and Schaffner (2013) demonstrated that the presence of *Listeria* spp. on a food-contact surface was a not a good predictor for the prevalence of *L. monocytogenes*. However, the detection of *L. monocytogenes* on food-contact surfaces is subjected to increased scrutiny by regulatory authorities. Hence, it is a major challenge for fresh produce processors to proactively seek *L. monocytogenes* in their facilities. Nevertheless, this seek & destroy approach and other efforts made by industry and regulatory agencies have helped in reducing *L. monocytogenes* contamination on RTE meat and poultry products (*136*).

2.4 Mushrooms and mushroom production processes

2.4.1 Taxonomy

Mushrooms are “macrofungus with a distinctive fruiting body that is large enough to be seen with the naked eye” (*142*). They are eukaryotes and belong to the Kingdom Fungi. Mushrooms are further classified into the following divisions: Ascomycota (the

largest division), Basidiomycota, Chytridiomycota, Zygomycota, and Glomeromycota (*44*, *221*). The most commercially cultivated edible fungal genera include *Agaricus* (button), *Lentinula* (shiitake), and *Pleurotus* (oyster) under the Basidiomycota division and Agaricomycotina subdivision. The most commonly known *Agaricus bisporus* mushrooms are white button, cremini, and portabello.

2.4.2 Commercial *Agaricus* mushroom production

Fresh white button mushrooms (*Agaricus bisporus*) comprised 90% of the US mushroom market during 2014 – 2015 (*208*). The nutritional benefits of mushrooms have been recognized during the past decade, resulting in increased demand. FIGURE 3 shows the total value and production of white button mushrooms in the US. Data from 2010 showed that Pennsylvania is the leading producer of *Agaricus* mushrooms in US accounting for 65% of the mushroom market, followed by California (FIGURE 4).

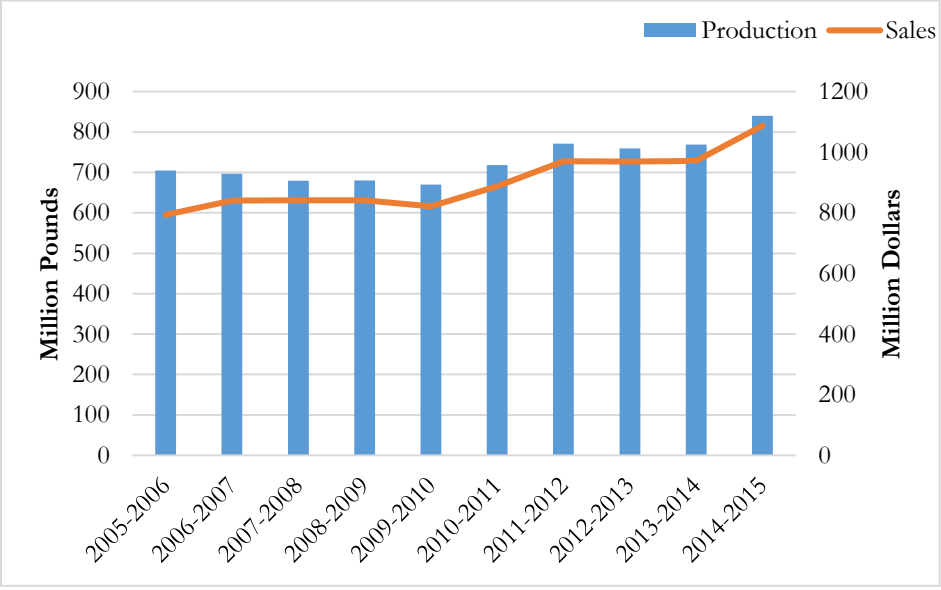

FIGURE 3: Total value of sales and production of *Agaricus* mushrooms in the United States. Adapted from USDA (2015).

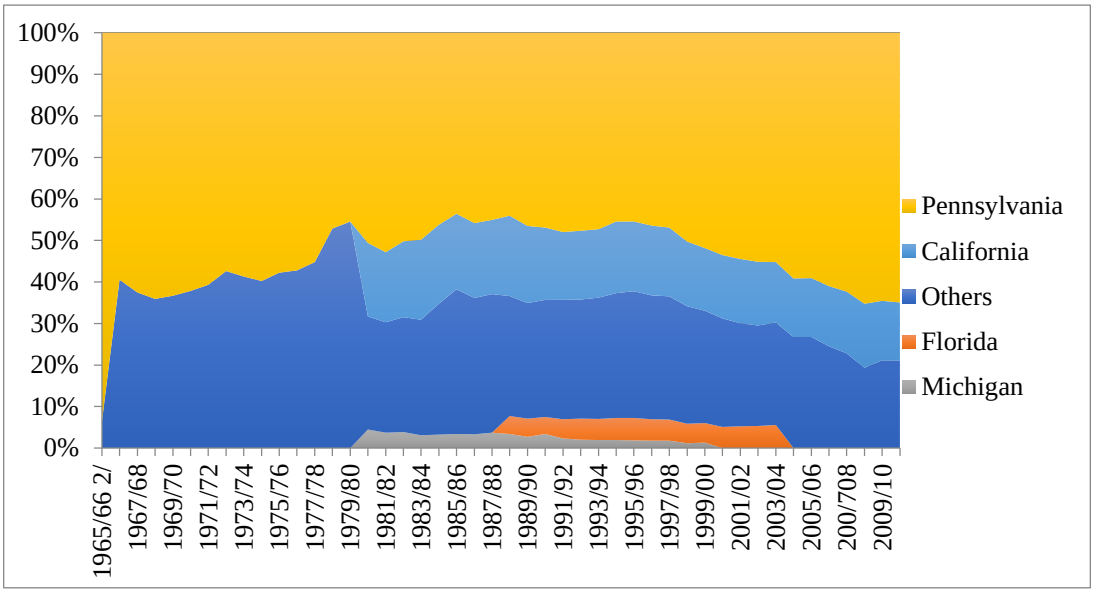

FIGURE 4: Mushroom production by different states in the United States**.** Adapted from USDA (2011).

2.4.3 Composition of *Agaricus* mushrooms

Mattila et al. (*139*) reported that 100 g of fresh white *Agaricus bisporous* mushrooms contain 92.3 g moisture and 7.7 g dry matter. The dry matter includes 4.5 g carbohydrates (1.5 g of dietary fiber), 2.1 g protein, 0.33 g crude fat, and 0.78 g ash (*139*). The major carbohydrates present in button mushrooms are chitin, glucose, mannitol, glycogen, and trehalose (*44*, *139*, *217*). The cell wall of mushrooms is primarily composed of chitin, an insoluble polymer made of N-acetyl-glucosamine units (*120*, *139*, *221*). Mushrooms contain 17 of 20 essential amino acids and are good sources of selenium, riboflavin, niacin, vitamin D, and the anti-oxidant, ergothioneine (*138*).

2.4.4 Pre-harvest operations

Mushroom farming consists of the following six processing steps which take 14 weeks to complete production: phase I composting, phase II composting, spawning, casing, pinning, and harvesting (*183*). These processes are briefly described below. More detailed information on these steps has been published by Beyer (2003) and Royse and Beelman (2007).

2.4.5 Phase I and II composting

Phase 1 composting involves preparation of raw materials for mushroom production. The raw materials are collectively called compost, which consists of straw-bedded horse manure, hay or wheat straw, poultry manure, corncobs, cottonseed hulls, cocoa bean hulls, nitrogen supplements, and gypsum (*13*, *183*). After preparation, the compost is then pasteurized in the phase II composting process. Pasteurization is mainly performed to eliminate vegetative fungal pathogens that can cause diseases on mushrooms or compete with mushrooms for nutrients resulting in a lower yield.

2.4.6 Spawning and casing

The pasteurized and cooled compost is mixed with grains inoculated with *Agaricus bisporus* mycelium, a mixture known as spawn. The spawn grows and forms hyphae which then develop into a filamentous mycelium. After complete colonization of the spawn, casing soil (sphagnum peat moss and limestone) is added as a thin layer to the compost surface. The main functions of casing soil are to retain water to support

mushroom growth, protect the compost from drying, and provide structural support for developing mushrooms (*183*).

2.4.7 Pinning and harvesting

After casing, mushroom pins start to form on the surface of the casing soil. Pins develop to form mature mushrooms within 18 to 21 days after which they are ready to be harvested. White button and cremini mushrooms are harvested with their veils intact, whereas portabella mushrooms are harvested with their veils open. Harvesting occurs in 7 to 10 day intervals and is referred to as a flush, break, or bloom. Harvesting is performed mainly by hand, but mechanical means are also used. Once mushrooms are harvested, they are sent to a packaging facility for processing. After completion, the spent mushroom compost is pasteurized by injecting steam into growing rooms and is generally not reused for further cropping due to lack of essential nutrients for growth of mushrooms (*13*, *183*).

2.4.8 Post-harvest processing operations

Post-harvest fresh mushroom processing operations include washing, slicing, and packaging. Washing of mushrooms is generally performed to remove the casing soil particles. A study by Beelman and Duncan (1999) summarized the effects of different wash solutions on the quality of mushrooms. Briefly, the washing procedure comprises a multistage process that includes washing mushrooms with an electrolyzed-oxidized water with a minimum pH of 9.0 followed by a neutralizing wash with organic acids supplemented with a browning inhibitor (*9*). This washing procedure was shown to

reduce spoilage microorganisms and improve overall acceptance of mushrooms by inhibiting enzymatic browning. Fresh *Agaricus* mushrooms are commercially available in either sliced or whole form. Depending on the product demand, the mushrooms can be sliced or packaged as whole in polystyrene trays and overwrapped with perforated plastic film for retail display.

2.4.9 Microbiology of fresh mushrooms

Fresh mushrooms, compost, and casing soil have been reported to contain high microbial populations (*44*, *57*, *226*). Fresh mushrooms have a pH of 6.8 and water activity of 0.99 (*123*), suggesting an optimum environment for the growth of microorganisms. Total aerobic plate counts of *Agaricus* mushrooms range from 6 to 8.8 log CFU/g, and yeast and mold from 1.8 to 3.3 log CFU/g (*57*, *219*). The two most predominant bacterial genera identified in mushrooms are *Pseudomonas* and *Flavobacterium* (*57*). Other bacterial genera including *Chryseobacterium, Coryne*form, *Lactobacillus*, and *Pediococcus* have also been detected in fresh mushrooms (*44*). A study by Chikthimmah (2006) showed that casing soil is also a rich source of microorganisms including *Pseudomonas*, *Pantoea*, *Streptomyces*, and *Penicillium.*

2.4.10 Sources of *L. monocytogenes* contamination in mushroom production environments

Growing, packing, and slicing environments could be potential sources of *L. monocytogenes* contamination of mushrooms. A study by Viswanath et al. (*222*) investigated pre-harvest environmental sources of *Listeria* spp., including *L.*

monocytogenes, on a small-scale *Agaricus bisporus* mushroom farm. The study revealed that 15.8% of environmental samples were positive for *Listeria* spp. However, *L. monocytogenes* (serotype 4a, lineage III) was isolated in only 1.6% of samples, all taken from the growth substrate ingredient receiving and storage area (*222*). Horse and poultry manures are widely used to prepare *Agaricus* mushroom growth substrate and have been suggested as potential sources of contamination with *L. monocytogenes* and other pathogens. However, a study by Weil et al. (*226*) demonstrated that the industry standard 6-day commercial mushroom growth substrate composting process, which includes a thermal treatment of 60°C for 2 h, was sufficient to achieve at least a 7-log reduction of *L. monocytogenes*.

In contrast to the results of the *A. bisporus* farm study (*222*), another study conducted on four *Flammulina velutipes* (enokitake) production facilities revealed that *L. monocytogenes* was present in 18.6% of samples with the majority of isolates present after the spawning area. A mycelium stimulating machine was identified as the source of *L. monocytogenes* contamination in *F. velutipes* facilities (*38*). The variation in distribution of *L. monocytogenes* at different mushroom production stages between these two studies might be due to differences in processing methods and practices employed in these facilities.

Another possible source of pre-harvest *L. monocytogenes* contamination on mushrooms is the casing soil (sphagnum peat moss and limestone) which is typically applied to the surface of the mushroom substrate to promote mycelium growth. A previous survey by Koeslag (2015) showed that 3.3% of the sugar beet lime samples (a component of casing soil) were positive for *L. monocytogenes.* But, studies conducted by

Chikthimmah et al. (*45*) and O'Patchen and LaBorde (*158*) demonstrated that non-sterilized sphagnum peat moss suppressed the growth and survival of *L. monocytogenes* and *Salmonella* to below detectable levels after 14 days in casing soil. These studies also demonstrated a rapid die-off of *L. monocytogenes* between application of the peat layer and emergence of mushrooms (*45*, *158*).

In contrast, another study by Leong et al. (*122*) showed that casing soil allowed the survival of *L. monocytogenes* for 20 d at 20°C, but did not support the growth of *L. monocytogenes*. However, this study did not inoculate the casing soil with *A. bisporus* spawn. This is important because the discrepancy in results might be partially due to the utilization of nutrients by mushrooms from the compost and casing soil, thus allowing fewer available nutrients for *L. monocytogenes* growth and survival. Nevertheless, these studies suggest that the compost and casing soil are less likely to be sources of *L. monocytogenes* contamination on mushrooms.

2.5 Conclusions and research objectives

Fresh produce outbreaks and recalls associated with *L. monocytogenes* contamination have been increased recently, resulting in increased economic loss and decreased public health. Recalls on fresh commercially grown mushrooms due to *L. monocytogenes* contamination have raised concerns in the fresh mushroom industry. Additionally, mushroom growers and processors are facing increased scrutiny from wholesale buyers for evidence of proactive control measures to prevent pre- and post-harvest contamination of products with *L. monocytogenes*.

Previous studies from the mushroom farm environment suggest that pre-harvest practices are less likely sources of *L. monocytogenes* contamination in mushrooms. However, the contamination risk of *L. monocytogenes* in the mushroom processing environment is unknown. Previous fresh produce outbreak tracking studies reported that the processing environment is the major source of *L. monocytogenes* contamination. Nevertheless, the prevalence and persistence of *L. monocytogenes* clones in a commercial fresh-cut produce processing environment, including mushrooms, have not yet been determined. Therefore, **the first objective of this research was to determine the prevalence and persistence of *Listeria monocytogenes* in a commercial fresh-cut mushroom slicing and packaging facility.**

Specific aim 1: Conduct a longitudinal study to determine the prevalence of *Listeria* spp. and *L. monocytogenes.*

Specific aim 2: Identify the persistent and transient *L. monocytogenes* subtypes using multi-virulence-locus sequence typing.

For development of effective control and elimination strategies for persistent *L. monocytogenes* contamination, it is important to understand the behavior of persistent subtypes under different environmental conditions. Variations in response to different environmental factors or stresses between persistent and transient subtypes might explain the clonal selection and niche adaptation in the mushroom processing facility. Therefore, **the final objective was to evaluate factors causing predominance and persistence of *L. monocytogenes* subtypes isolated from the mushroom processing facility.**

Specific aim 1: Determine differences in tolerance or resistance to sanitizer between persistent and transient *L. monocytogenes* subtypes.

Specific aim 2: Evaluate variations in firmly adhered populations on stainless steel and concrete among persistent and transient *L. monocytogenes* subtypes.

Specific aim 3: Determine differences in growth rate and survival between persistent and transient *L. monocytogenes* subtypes at different mushroom nutrient concentrations.

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Chapter 3

Predominance and distribution of a persistent *Listeria monocytogenes* clone in a commercial fresh mushroom processing environment

Running title: *Listeria* in a mushroom processing environment

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3.1 Abstract

A longitudinal study was conducted to determine the prevalence of *Listeria* spp. in a commercial fresh mushroom slicing and packaging environment. Samples were collected at 3 different sampling periods within a 13-month time interval. Of the 255 environmental samples collected, 18.8% tested positive for *L. monocytogenes*, 4.3% for *L. innocua* and 2.0% for *L. grayi*. *L. monocytogenes* was most often found on wet floors within the washing and slicing, and packaging areas. Each of the 171 *L. monocytogenes* isolates found in the environment could be placed into 1 of 3 different serotypes; 1/2c was the most predominant (93.6%) followed by 1/2b (3.5%) and 1/2a (2.9%). Of 58 isolates subtyped using multi-virulence-locus sequence typing (MVLST), all 1/2c isolates were identified as virulence type 11 (VT11), all 1/2b isolates were VT105, and 1/2a isolates were either VT107 or VT56. VT11 was designated as the predominant and persistent clone in the environment because it was isolated repeatedly at numerous locations throughout the study. The overall predominance and persistence of VT11 indicates that it likely colonized the mushroom processing environment. Areas adjacent to the trench drain in the washing and slicing area and a floor crack in the packaging area may represent primary harborage sites (reservoirs) for VT11. Improvements made to sanitation procedures by company management after Period 2 coincided with a significant ($p \leq 0.001$) reduction in the prevalence of *L. monocytogenes* from 17.8% in Period 1 and 30.7% in Period 2 to 8.5% in Period 3. This suggests that targeted cleaning and sanitizing procedures can be effective in minimizing the occurrence of *L. monocytogenes* contamination in processing facilities. Additional research is needed to

understand why VT11 was predominant and persistent in the mushroom processing environment.

3.2 Introduction

Listeria monocytogenes is a pathogen of concern to food processors, regulators and consumers because of its ubiquitous nature, ability to grow at refrigerated temperatures and high case-fatality rate of 20-30% (*44*). *L. monocytogenes* strains differ in their ability to cause disease, outbreaks and epidemics. Among 13 serotypes of *L. monocytogenes*, 4b, 1/2a and 1/2b account for more than 95% of human listeriosis cases (*5*, *28*). Within these serotypes, most major outbreaks are caused by a limited number of epidemic clones (ECs): ECI, ECII, ECIV and ECV in serotype 4b, ECIII and ECVII in serotype 1/2a, and ECVI in serotype 1/2b (*17*, *37*).

Post-processing contamination of ready-to-eat (RTE) foods with *L. monocytogenes* is a continuing concern to the food industry (*45*). Outbreaks of listeriosis have historically been most often associated with raw or processed dairy products and RTE sliced deli meats (*22*). More recently, *L. monocytogenes* has become a growing problem in the fresh produce industry. Recent listeriosis outbreaks linked to the consumption of fresh fruits and vegetables include diced celery in 2010 (*23*), cantaloupe in 2011 (*4*), mung bean sprouts in 2014 (*6*) and caramel-coated fresh apples in 2015 (*7*).

Contamination of RTE food products by *L. monocytogenes* is often attributed to the presence of persistent strains which have colonized processing facilities for months to decades (*26, 28, 59*). Persistence has been defined as “repeated isolation on different dates of *L. monocytogenes* strains that are subsequently identified as identical subtypes (as determined by phenotypic or genotypic methods)” (*22*). Persistent *L. monocytogenes* strains are thought to have adapted to the food processing environment through 1)

enhanced surface adherence and biofilm formation; 2) tolerance to sanitizers, heavy metals, osmotic shock and cold stress; and/or 3) resistance to phage attack (*22*, *29*, *38*, *45*, *49*, *54*). This suggests that mechanisms of persistence involve complex interactions between both biotic and abiotic factors present within the processing environment and genotypic/phenotypic aspects of the pathogen.

Fresh commercially grown mushrooms have not been implicated in any foodborne outbreaks to date. However, microbial surveys of fresh produce have reported the presence of *Listeria* spp. on commercially grown fresh mushrooms (*25*, *51*, *52*) and recalls of sliced mushrooms due to *L. monocytogenes* contamination have occurred recently (*8*, *9*, *10*, *11*, *55*). Studies have shown that *L. monocytogenes* is capable of growing on whole and sliced *Agaricus* mushrooms (*19*, *24*, *35*). Therefore, targeted and effective control measures must be put in place to prevent product contamination.

Reflecting recent trends within the entire produce industry, mushroom growers and processors are facing increased scrutiny from wholesale buyers for evidence that proactive control measures are in place to prevent pre- and post-harvest contamination of products with *L. monocytogenes* and other pathogens. It can be expected that upcoming regulations under the 2011 Food Safety Modernization Act [P.L. No. 111-353] will accelerate the need for research-based risk assessments on fresh produce.

The highly specialized process of growing *Agaricus* mushrooms on composted organic growth substrates has been described in detail by Beyer et al. (*3*). Pre-harvest environmental sources of *Listeria* spp., including *L. monocytogenes*, on a small-scale *A. bisporus* mushroom farm were investigated by Viswanath et al. (*57*). The study revealed that 15.8% of environmental samples were positive for *Listeria* spp. However, *L.*

monocytogenes (serotype 4a, lineage IIIA) was only isolated in 1.6% of samples, all taken from the growth substrate ingredient receiving and storage area (*57*). Horse and poultry manures are widely used to prepare *Agaricus* mushroom growth substrate and have been suggested as potential sources of contamination with *L. monocytogenes* and other pathogens. However, a study by Weil et al. (*58*) demonstrated that the industry standard 6-day commercial mushroom growth substrate composting process, which includes a thermal treatment of 60°C for 2 h, was sufficient to achieve at least a 7-log reduction of *L. monocytogenes*. Although there may be potential for *L. monocytogenes* contamination of organic peat typically applied on the surface of the growth substrate, previous inoculation studies in our laboratory demonstrated rapid die-off of *L. monocytogenes* between application of the peat layer and emergence of mushrooms (*18, 47*) .

To date, no studies have investigated the potential for post-harvest contamination in a commercial mushroom (*Agaricus bisporus*) slicing and packaging facility. Therefore, this study was conducted to investigate the prevalence, distribution, and potential routes of contamination of *Listeria* spp., including *L. monocytogenes,* in the commercial mushroom slicing and packaging environment. Multi-virulence-locus sequence typing (MVLST) was used to identify specific locations where persistent *L. monocytogenes* clones would present a higher risk for product contamination.

3.3 Materials and methods

Mushroom facility layout and processing steps. The facility surveyed in this study was constructed in 1947 for use as a commercial mushroom canning operation. In 2004, it was converted into a fresh mushroom slicing and packaging operation. During the course of this study, mushrooms (*A. bisporus*) were sourced from several farms within a 25 mile (40 km) radius of the facility. Two types of *A. bisporus* were packaged, the common white button mushroom (75%), and brown cremini and portabella mushrooms (25%). Approximately 50% of the mushrooms were sliced prior to packaging. Each day, the plant ran two 8-h production shifts followed by an 8-h sanitation shift.

For purposes of this study, the facility was divided into 5 zones (FIGURE 5). Upon receipt, mushrooms were immediately vacuum-cooled to 4°C and stored for processing and packaging in the staging area (Zone 1). For sliced mushroom products, baskets of cooled mushrooms were transported by hand trucks to the washing and slicing area (Zone 2) where they were loaded onto a receiving conveyor (A) which moved them onto the washer conveyor (B). Button mushrooms were passed through a multi-stage re-circulating proprietary washing system. After washing, mushrooms moved automatically onto the slicer conveyor (C) for alignment and slicing with a double bladed stainless steel disc slicer. The sliced mushrooms then moved by conveyor (D) into the packaging room (Zone 3) and then onto another conveyor (E) where workers weighed and filled them into polystyrene trays for overwrapping with perforated plastic film.

Button mushrooms to be sold unsliced moved from the receiving and staging area (Zone 1) directly onto conveyor F in the packaging area (Zone 3) for filling and overwrapping with perforated plastic film (FIGURE 5). Packaged sliced and whole button mushrooms were moved by conveyor (G) into the shipping area (Zone 4) for loading into refrigerated trucks. Non-processing areas which surrounded the processing areas such as a lunch and locker room, boiler room, dry warehouse, walk-in cooler, storage room and business office were considered as Zone 5 (FIGURE 5). All processing areas were kept continuously chilled and the average air temperatures measured over the 3 sampling periods were; Zone 1: 2.6 ± 0.6, Zone 2: 9.7 ± 0.8, Zone 3: 10.1 ± 2.4, and Zone 4: 3.0 ± 0.4°C.

According to company management, floors were cleaned each day after production ended with a chlorinated alkaline solution using a mechanical floor scrubber, after which they were sanitized with a quaternary ammonium compound (QAC) solution. Equipment was also cleaned with chlorinated alkaline solution, and, on alternate days, was sanitized with a commercial QAC solution or a peroxyacetic acid/hydrogen peroxide product. A QAC foam sanitizer was automatically applied every 30 min to floors near doors in the washing and slicing area (Zone 2) (FIGURE 5). Management stated that all cleaning and sanitizing products were applied according to label recommendations and that food-contact and non-food-contact surfaces were regularly tested for *Listeria* spp.

Sample collection. Company management granted access to the mushroom facility upon the condition that only non-food-contact environmental sites could be sampled as long as care was taken not to interfere with normal operations. Within these

limitations, samples were taken at points along the flow of the product from receiving to shipping. Sampling sites were initially chosen based on established knowledge of environmental conditions and niches known to support the survival and growth of *Listeria* spp. Priority was given to continuously wet areas adjacent to food-contact surfaces where product contamination is more likely to occur. Consideration was also given to observed worker and equipment traffic patterns. Sampling was conducted 3 times during this study; May 29, 2012 (Period 1), January 25, 2013 (Period 2), and June 27, 2013 (Period 3). A summary of the number of samples taken for each sampling period and descriptions of the sampling sites are shown in TABLE 2.

Pre-hydrated sterile Quick™ swabs, sponges, and sponge sticks immersed in neutralizing buffer (3M Microbiology, St. Paul, MN, USA) were used for surface sampling. Quick™ swabs were used for sampling 2 cm X 2 cm areas on equipment nuts, bolts and rollers. Sponges and sponge sticks were used to swab 30 cm X 30 cm areas on floors, wall, drain and wider equipment surfaces. Sponges and sponge sticks (4 cm wide) were also used to sample narrow cracks along an approximately 10-15 cm length. All samples were taken at least 3 h after the start of operations and transported on ice to the Department of Food Science at The Pennsylvania State University where they were held at 4^{o}C until analyzed the next day.

Detection and isolation of *Listeria* spp. Samples were enriched in 100 mL (sponge and sponge stick) or 10 mL (swab) modified University of Vermont Medium (mUVM) (BD Diagnostic Systems, Franklin Lakes, NJ, USA). The bags were hand-massaged for 10-15 s before incubation at 30°C for 48 h. Enriched samples were tested

for the presence of generic *Listeria* spp. using the mini-VIDAS system (BioMérieux, Marcy-l'Étoile, France). For sampling Period 1, presumptive *Listeria* spp. samples were streaked onto Modified Oxford agar plates (MOX) (BD Diagnostic Systems) and incubated at 35°C for 48 h. Presumptive *Listeria* isolates were visually identified as grayish-white colonies surrounded by zones of blackening. For further confirmation, 4 typical *Listeria* spp. colonies from MOX plates (except one sample that contained only 2 colonies) were streaked onto RAPID' *L. mono*™ plates (Bio-Rad Laboratories, Hercules, CA, USA) and incubated at 35°C for 24 h. In some samples taken during sample Period 1, non-*Listeria* background microbiota showed false-positive reactions on MOX plates. Therefore, this step was eliminated in subsequent sampling periods, and instead, presumptive *Listeria* spp. samples from mini-VIDAS were directly streaked onto RAPID' *L. mono*™ plates. To ensure pure cultures, presumptive *Listeria* spp. colonies from RAPID' *L. mono*™ plates were streaked onto tryptic soy agar plates (BD Diagnostic Systems) containing 0.6% yeast extract (BD Diagnostic Systems) (TSAYE) and incubated at 35°C for 24 h. Isolates from TSAYE plates were used to make 15% glycerol stock cultures and were stored at -20°C prior to further analysis.

Confirmation of *Listeria spp.* and *L. monocytogenes.* Presumptive *Listeria* spp. isolates were confirmed by multiplex PCR (*14*). Prior to multiplex PCR, DNA was extracted using Ultraclean® Microbial DNA Isolation kits (Mo Bio Laboratories Inc., Carlsbad, CA, USA). PCR was performed with Multiplex PCR Plus kits (Qiagen Inc., Valencia, CA, USA) using *iap* and *lmo2234* primers specific for *Listeria* spp. and *L.*

monocytogenes, respectively, as previously described (*14*). All confirmed *Listeria* isolates were identified to species level using API *Listeria* test strips (BioMérieux).

***L. monocytogenes* serogrouping and serotyping.** Confirmed *L. monocytogenes* isolates were serogrouped using the multiplex PCR procedure of Doumith et al. (*21*). In order to differentiate *L. monocytogenes* from other *Listeria* spp., the procedure was slightly modified by replacing *prs* primers with *lmo2234* primers (*14*). All other gene targets and PCR conditions were followed as previously described (*21*). For each positive *L. monocytogenes* sample, one isolate from each serogroup present was sent to the Centers for Disease Control and Prevention (CDC) for serotyping using somatic and flagellar *Listeria* antisera (Denka Seiken Co. Ltd, Tokyo, Japan). Serotyping of *L. monocytogenes* isolates was performed according to the manufacturer's instructions.

Subtyping of *L. monocytogenes* isolates using multi-virulence-locus sequence typing (MVLST). MVLST, previously shown to accurately differentiate epidemic, outbreak and non-outbreak clones of *L. monocytogenes* (*15*, *37*, *60*), was used to subtype *L. monocytogenes* isolates. One isolate from each positive sample was selected for MVLST analysis. Multiple isolates were selected when samples contained mixed serotypes. The following new *dal* primers were designed using Primer BLAST from the National Center for Biotechnology Information: F (5-3') - AGCGAAAGAAGCTGGAGCAA and R (5'-3') – GCAAGGGCTGGTTTAAGCTC. These primers were tested against reference isolates F2365 (4b), J1022 (1/2c), L2626 (1/2a), and L2624 (1/2b) for an amplification size of 570 bp. Amplicons were cleaned

using ExoSAP-IT® (Affymetrix, Santa Clara, CA, USA) and sent to The Genomics Core facility (The Pennsylvania State University, University Park Campus, PA, USA) for forward and reverse sequencing. The gene sequences were aligned, concatenated and analyzed using molecular evolutionary genetic analysis software (MEGA 6.0, Tempe, AZ, USA). All confirmed *L. monocytogenes* isolates from the mushroom processing facility were compared with 96 known virulence types (VTs) from author Knabel's MVLST database collection. The database includes outbreak and non-outbreak isolates from humans, foods, animals, and processing environments (*36*). An unrooted Neighbor-Joining tree with bootstrap values of 1000 was constructed to allow ease of subtype comparison between isolates.

Nucleotide accession numbers. Gene sequences of confirmed *L. monocytogenes* isolates were deposited into GenBank under accession numbers; KT199758 through KT199815 (*clpP*), KT199816 through KT199873 (*dal*), KT199874 through KT199931 (*inlB*), KT199932 through KT199989 (*inlC*), KT199990 through KT200047 (*lisR*), and KT200048 through KT200105 (*prfA*).

Statistical analysis. Fisher's exact test was used to analyze differences in the prevalence of *L. monocytogenes* and other *Listeria* spp. based on sampling periods using SAS statistical software (SAS Institute Inc., Cary, NC, USA). Data from all sampling sites during Periods 1 and 2 were grouped and compared with Period 3 to determine the effect of sanitation practice changes made by company management after Period 2. Because our method for selecting sample sites was biased toward the packaging area

(Zone 3) and the washing and slicing area (Zone 2), a statistical analysis for zone effect was not conducted.

3.4 Results

Prevalence of *Listeria* spp. The prevalence of *Listeria* spp. within each zone and sampling period is shown in TABLE 3. Over the course of the 13-month study, 255 environmental samples were taken at 98 sites within the mushroom processing environment. Among all samples taken, 61 (23.9%) were confirmed positive for *Listeria* spp. *L monocytogenes* was found in 48 samples (18.8%), *L. innocua* in 11 samples (4.3%), and *L. grayi* in 5 samples (2.0%). No other *Listeria* spp. were found during all sampling periods. In 3 samples, more than one *Listeria* spp. was found. Wall, ceiling and door samples in all zones and at all sampling periods were negative for *Listeria* spp.

One or more *Listeria* spp. were found in each zone during at least one sampling period except the non-processing areas (Zone 5). We observed that *L. monocytogenes* was more frequently detected in washing and slicing (30.2%) (Zone 2) and packaging areas (18.5%) (Zone 3), compared to all other zones in the facility. In contrast, only 2.6% of samples from the receiving and staging area (Zone 1) were positive for *L. monocytogenes* and none were positive in the shipping (Zone 4) and non-processing areas (Zone 5). *L. innocua* was infrequently detected in Zones 2 and 3. It was only found on floor samples adjacent to the slicer and a door, and on a support bar and a floor mat near conveyor A. In contrast, *L. innocua* was predominantly found in the receiving and staging area (Zone 1) and shipping area (Zone 4). *L. grayi* was only detected at 5 sampling

locations, most often in Zones 2 and 3 in areas adjacent to the slicer in the washing and slicing area (Zone 2) and conveyor F in the packaging area (Zone 3).

A statistical analysis was conducted to determine if prevalence rates for individual *Listeria* spp. were significantly affected by sampling periods. The grouped prevalence rate for *L. monocytogenes* across all zones during Period 1 (17.8%) and Period 2 (30.7%) was significantly ($p \leq 0.002$) higher than during Period 3 (8.5%). In contrast, there were no significant ($p > 0.05$) differences between sampling periods for the prevalence of *L. innocua* or *L. grayi* across all zones.

Distribution of *L. monocytogenes.* Descriptions of sampling sites that were positive for *L. monocytogenes* during at least one sampling period (N = 30) are shown in TABLE 3. A subset of locations (N = 15) were positive for *L. monocytogenes* during more than one sampling period. In general, *L. monocytogenes* was detected more often on floors, floor cracks, floor crevices, and floor-contact materials (floor mats, equipment wheels, hand truck wheel, stairs, a trench drain) (N = 26) than on non-food-contact equipment framework surfaces and conveyors (N = 4) (TABLE 3).

During the study, some sites were eliminated because equipment had been removed and therefore was not available for resampling. In some instances, additional suspected harborage sites were included in the sampling plan after Period 1. Therefore, only sites that were sampled during each of the 3 periods (N = 63) were used to directly compare the frequency of *L. monocytogenes* contamination at different locations within the facility (FIGURE 6). Sites where *L. monocytogenes* was never detected are shown in open circles (N = 43). These sites were primarily in the

receiving and staging area (Zone 1), near the whole mushroom packaging area (Zone 3), the shipping area (Zone 4) and the non-processing area (Zone 5) (FIGURE 6). Sites testing positive for *L. monocytogenes* once (grey filled circles), twice (black filled circles) or 3 times (black filled star) (N = 20) are also displayed in FIGURE 6. It is apparent that sites positive for *L. monocytogenes* during only 1 of the 3 sampling periods (N = 9) were mainly located under and around conveyor C in the washing and slicing area (Zone 2). Sites where *L. monocytogenes* was found during 2 of the 3 sampling periods (N = 9) were also concentrated around the washing and slicing lines in Zone 2, including under conveyor D where the exposed sliced mushrooms move into the packaging area (Zone 3) (FIGURE 6). *L. monocytogenes* was positive during all 3 sampling periods at only 2 sites; a floor crack at the junction of the trench drain metal grate and the concrete floor (WS-12) in the washing and slicing area (Zone 2) and a floor crack under conveyor D (P-1) in the packaging area (Zone 3) (FIGURE 6 and TABLE 4).

Identification of *L. monocytogenes* serogroups and serotypes. Overall, 171 *L. monocytogenes* isolates, obtained from 48 positive samples, were serogrouped by multiplex PCR. Of these, 93.6% belonged to serogroup 1/2c or 3c, 3.5% were serogroup 1/2b or 3b, and 2.9% were serogroup 1/2a or 3a (data not shown). Of the 49 *L. monocytogenes* isolates sent to CDC, all 38 from serogroup 1/2c or 3c were identified as serotype 1/2c, all 6 from serogroup 1/2b or 3b were serotype 1/2b, and all 5 from serogroup 1/2a or 3a were serotype 1/2a. The 1/2c serotype was found in 28 of 30 *L. monocytogenes* positive sites (TABLE 4). In contrast, serotype 1/2b was found at only 2

sites (WS-1, WS-22) while serotype 1/2a was found at 4 sites (RS-1, WS-11, WS-13, P-1). Co-detection of serotype 1/2c with 1/2b or 1/2a occurred at only 1 (WS-1) and 3 (WS-11, WS-13, P-1) sites, respectively (Table 3).

***L. monocytogenes* virulence types.** MVLST subtyping was performed on all serotype 1/2a (N = 5) and 1/2b (N = 6) isolates, and one serotype 1/2c isolate from each of the *L. monocytogenes* positive samples (N = 47). FIGURE 7 shows the phylogenetic relationships between the isolates found in the mushroom processing environment and 96 known VTs from author Knabel's MVLST database collection. Based on similarity in 6 virulence gene sequences of 58 mushroom facility isolates, 81.03% were VT11, 10.35% were VT105, 5.17% were VT107, and 3.45% were VT56. All 1/2c isolates were classified as VT11. Serotype 1/2a isolates were classified as VT56 (ECVII) or a new VT, VT107. Serotype 1/2b isolates obtained on 2 different sampling occasions had identical virulence gene sequences and were also assigned a new VT, VT105. All VT105 isolates had an indel of 141 bp in *inlB*, and *dal* failed to amplify using previously designed primers (*60*). Therefore, newly designed *dal* primers were used to amplify *dal* for this VT. No truncations were found in any tested internalins or other virulence genes in VT107 and VT56 in 1/2a serotype, and VT11 in 1/2c serotype.

Predominant and persistent *L. monocytogenes* virulence types. The results in Table 3 reveal that VT11 was found in 96% (46/48) of *L. monocytogenes* positive samples. In contrast, VT107, VT105, and VT56 were found in only 6.3, 4.2, and 2.1% of *L. monocytogenes* positive samples, respectively. The total frequencies added up to more

than 100% because VT11 was co-detected with VT107 in 4 samples and with VT105 in 1 sample. These results revealed that VT11 was the predominant clone in the facility environment.

VT11 was found during more than one sampling period at 15 of 30 *L. monocytogenes* positive sites and was the only VT isolated during all 3 sampling periods; near the trench drain in Zone 2 (WS-12) and a floor crack in Zone 3 (P-1) (TABLE 4). In contrast, VT105 was found at 2 locations (WS-1, WS-22); however, each during different sampling periods. VT107 was detected at 3 locations (WS-11, WS-13, P-1) during Period 2, and VT56 was found at one location (RS-1) also during Period 2 (TABLE 4). If we modify the general definition of persistence from Ferreira et al. (*22*) to more specifically designate persistent VTs as those isolated at the same sampling site during at least 2 of 3 sampling periods, then VT11 can be considered not only the predominant clone, but also the only persistent clone in the mushroom processing environment. Using this modified definition of persistence, the other VTs; VT105, VT107, and VT56 would be considered transient clones.

3.5 Discussion

The results showed that the predominant *Listeria* spp. found in the commercial mushroom processing environment was *L. monocytogenes*. The prevalence rate of *L. monocytogenes* on non-food-contact surfaces taken over all zones and sampling periods (18.8%) is similar to that reported for other facilities that process cheese (19.5%) (*50*),

catfish (18.4%) (*12*), cold-smoked rainbow trout (18.7%) (*1*), pork (12.4%) (*33*), and non-*Agaricus* enoki mushrooms (*Flammulina velutipes*) (18.6%) (*13*).

The greater frequency at which *L. monocytogenes* was isolated compared to other *Listeria* spp. is in contrast to a recent study in a small-scale *Agaricus* mushroom farm, where *L. innocua* was detected more frequently (10.3%) than *L. monocytogenes* (1.2%) (*57*). Differences in the prevalence of *L. monocytogenes* and *L. innocua* between the two studies may be related to how each species responds to different environmental conditions or stresses encountered in production versus processing environments (*44*). The lower prevalence of *L. monocytogenes* in the small-scale mushroom farm might be due to the fact that only whole mushrooms were harvested and packed. Therefore, higher levels of moisture and mushroom debris associated with washing and slicing operations were not present in the small-scale farm.

The above correlation between water, food and *Listeria* contamination coincides with our observation of the environmental conditions in the processing facility during this study. At each visit, we observed that floors and equipment in Zone 2 appeared continuously wet from drainage and splash from the washer and slicer. In Zone 3, floors were sporadically wet due to employee and equipment traffic. Mushroom debris that had fallen from the slicer and conveyors was observed on floors and equipment support structures in Zones 2 and 3. Floors in Zones 1, 4 and 5 appeared dry with no visible water during each visit.

In addition to the above, restrictions on the movement of employees and equipment were not observed. Packaging line workers moved to and from lunch and locker rooms through the washing and slicing area (Zone 2) and to their work stations in

the packaging area (Zone 3) at the beginning and end of the work day, and during lunch time and mid-shift morning and afternoon breaks. Other workers in the receiving and staging area (Zone 1) were observed moving boxes and trays of unprocessed mushrooms into Zones 2 and 3 using hand trucks. Although office employees had free access to processing areas during the day, movement between Zone 5 and the rest of the facility was infrequent. Production supervisors, maintenance, and quality control workers were observed moving freely throughout the facility.

The high prevalence of *L. monocytogenes* in Zone 2 and in part of Zone 3 (Table 2) is concerning because other studies have shown increased risk of product contamination when the processing environment is contaminated with *L. monocytogenes* (*2, 53*). The extensive amount of water used for mushroom washing and mid-shift clean up, the continuous presence of mushroom debris in these zones, and unrestricted movement of workers and equipment all likely contributed to the high prevalence of *L. monocytogenes* in these zones. Other studies in meat, poultry, dairy and fish processing facilities have similarly shown that floor drains, wet processing areas, and worker and equipment traffic contribute to increased prevalence of *L. monocytogenes* (*2, 26, 39, 43, 53*, *54*). Detection of *L. monocytogenes* in Zones 2 and 3 during all 3 of the sampling periods (FIGURE 6) and our observations of frequent employee and hand truck traffic indicate that these are high priority areas for sanitation improvements.

After Period 2, we presented our findings to company management and offered general recommendations for controlling *Listeria* in the processing facility. These included 1) sealing floor cracks and crevices to reduce the number of *L. monocytogenes* harborage sites (reservoirs) that may decrease cleaning and sanitizing efficiency; 2)

alternating sanitizer types to minimize development of resistant/tolerant strains; and 3) eliminating the use of power hoses during production runs to prevent contamination due to dispersed aerosols.

We returned to the facility for a third sampling period approximately 3 months later at which time company management reported to us the following changes in practices and policies; 1) QAC floor sanitizer was replaced with another product that contained both QAC and hydrogen peroxide, although no changes were reportedly made to the equipment cleaning and sanitizing chemicals; 2) a time-release granulated QAC product was spread on the floor twice each week; 3) a power hose used for cleaning floors and equipment was removed and sanitation workers were instructed to minimize aerosol generation by manually cleaning floors and equipment with brushes and low pressure hoses; and 4) maintenance workers began the process of filling and sealing floor cracks and larger crevices with a cement sealer.

Before Period 3 sampling began we were able to visually confirm the presence of granulated QAC deposited on floors in the processing and packaging areas (Zones 2 and 3). We also observed that a crack adjacent to the floor drain had been filled, although it was not the same crack that we had sampled during previous sampling periods. The crevice next to the hand washing station where *L. monocytogenes* was detected during the previous two sampling periods (WS-17) had been filled with cement. It is notable that *L. monocytogenes* was not detected at this location during Period 3 (TABLE 4). The significant decrease in the prevalence of *L. monocytogenes* during sample Period 3 (TABLE 3) may be at least partly explained by the company implementing one or more of the recommended sanitation policy and procedure changes. Improvements in sanitation

procedures and re-training of employees have previously been shown to be effective in decreasing the prevalence of *L. monocytogenes* in food processing environments (*31*, *32*, *54*). While the significant reduction in the prevalence of *L. monocytogenes* after changes in sanitation procedures is encouraging, its continued presence during Period 3 indicates that harborage sites in the floor had not been completely eliminated in the facility.

All confirmed *L. monocytogenes* isolates were serogrouped into one of the four major serogroups: 1/2a or 3a in serogroup I.1, 1/2c or 3c in serogroup I.2, and 1/2b or 3b in serogroup II.2; however, 1/2c was the most predominant serotype. This predominance may be due to the differences in gene expression between these serotypes when exposed to different types of stressful conditions which may contribute to differences in prevalence of *L. monocytogenes* serotypes in this environment (*48*). Further subtyping using MVLST showed that VT11 was the predominant *L. monocytogenes* clone within the mushroom processing facility. MVLST has been shown to be an excellent tool for investigating the molecular epidemiology of *L. monocytogenes* because it possesses both high discriminatory power (*15*, *60*) and high epidemiologic concordance (*16*). As a result, MVLST was able to differentiate numerous outbreak clones, epidemic clones, and non-outbreak clones (*15*, *30*, *37*, *60*). Consistent with this, in the present study MVLST successfully identified multiple sites within specific areas of the mushroom processing environment (Zones 2 and 3) where *L. monocytogenes* VT11 was persistent (TABLE 4). Other studies have also shown that the occurrence of persistent subtypes varies within specific areas of processing facilities (*20*, *31*, *40*, *46*). For example, a previous study had shown that three persistent ribotypes of *L. monocytogenes* isolated from fish proccessing

plants over a one year period were never detected in the raw fish handling area, but were found in processing areas (*31*).

Interestingly, only two sites (WS-12, P-1) were positive for VT11 during all 3 sampling periods, indicating possible primary harborage sites (reservoirs) for this VT (Table 3). Preliminary data indicated that VT11 was not detected from pre-operative floor samples (N=11) during Period 3 after sanitation improvements were made (data not shown). However, VT11 was detected 3 h after the beginning of operations at sites WS-12 and P-1. We hypothesize that *L. monocytogenes* VT11 survived below the floor surface within the porous concrete matrix where a continuous supply of water and nutrients was available and where VT11 was protected from intermittent and short-duration contact with surface cleaning and sanitizing chemicals. Deeply embedded *L. monocytogenes* may have moved to the surface during operations due to continuous exposure to water, and were then detected by surface swabbing. The ability of VT11 to predominate and persist at these 2 harborage sites suggests that it may have adapted to these ecological niches. Possible reasons for predominance and persistence of VT11 at these sites include: 1) more efficient utilization of mushroom nutrients; 2) ability to form dense biofilms; 3) tolerance to cleaners and sanitizers regularly used in the facility; and/or 4) competitive advantages over resident microbiota.

The VT11 clone of *L. monocytogenes* was previously found in meat processing environments and RTE meat products (*42*). While it has not been traced to any foodborne outbreaks, it was associated with one reported case of human listeriosis (*36*). On the other hand, VT105, which was isolated at 2 sites in the mushroom processing facility on 2 separate sampling periods, is closely related to ECVI (FIGURE 7) which was one of the

clones linked to the 2011 cantaloupe outbreak (*37*). MVLST data showed that ECVI has a full-length *inlB*, whereas VT105 had an indel of 141 bp in *inlB.* VT107 differs from ECIII by only 4 single nucleotide polymorphisms (SNPs): 2 SNPs in *inlC* and 2 SNPs in *inlB* (FIGURE 7). ECIII was responsible for a listeriosis case associated with the consumption of turkey franks in 1988 and an outbreak associated with deli meats in 2002 (*56*). VT56 (ECVII) was traced to listeriosis outbreaks attributed to whipping cream during 2000 in Canada and cantaloupe during 2011 in US (*37*). Therefore, it is critical that the mushroom industry conduct routine environmental testing for the occurrence of *Listeria* spp. in their processing facilities and take corrective actions to eliminate them. Also, further research is needed to determine the serotype diversity in other commercial mushroom processing facilities and to demonstrate the true virulence potential of *L. monocytogenes* clones found in these facilities.

This study is in agreement with earlier studies that demonstrated the presence of persistent *L. monocytogenes* clones in meat, fish, and dairy food production and processing facilities. While some studies have tracked product contamination to strains on incoming raw materials and raw material receiving area, we did not detect VT11 in the raw material receiving environment (Zone 1) during this study. Moreover, persistent strains within food processing environments are thought to be the likely cause of most instances of *L. monocytogenes* product contamination (*41*). Persistent *L. monocytogenes* clones existing as biofilms on food-contact surfaces have also been reported as causing product contamination (*34*). Although we cannot speculate on the extent to which VT11 or other *L. monocytogenes* clones were persistent on food-contact surfaces such as

mushroom slicers and conveyors, these sites should be included in the company's *Listeria* environmental monitoring program.

To our knowledge, this is the first longitudinal tracking study conducted in a fresh produce processing plant. MVLST successfully identified a predominant and persistent clone of *L. monocytogenes* (VT11) at critical areas adjacent to food-contact equipment and exposed mushrooms. Risks for product contamination are highest where there are deficiencies in sanitation practices. Sanitation improvements made between Periods 2 and 3 were effective in significantly reducing the prevalence of *L. monocytogenes*, however eradication was not complete. In particular, contamination with VT11 near the trench drain and a floor crack is an indication that further progress is needed to eliminate hard to clean harborage sites on the floors and drains, and to restrict employee and equipment traffic through these areas.

Further research is needed to understand the mechanisms responsible for the predominance and persistence of VT11 in this facility. Such findings might explain why certain *L. monocytogenes* clones persist in food processing facilities and others are transient. This information might then be used to provide guidance in designing targeted intervention measures that prevent *L. monocytogenes* contamination in fresh produce facilities as well as various other RTE food processing facilities.

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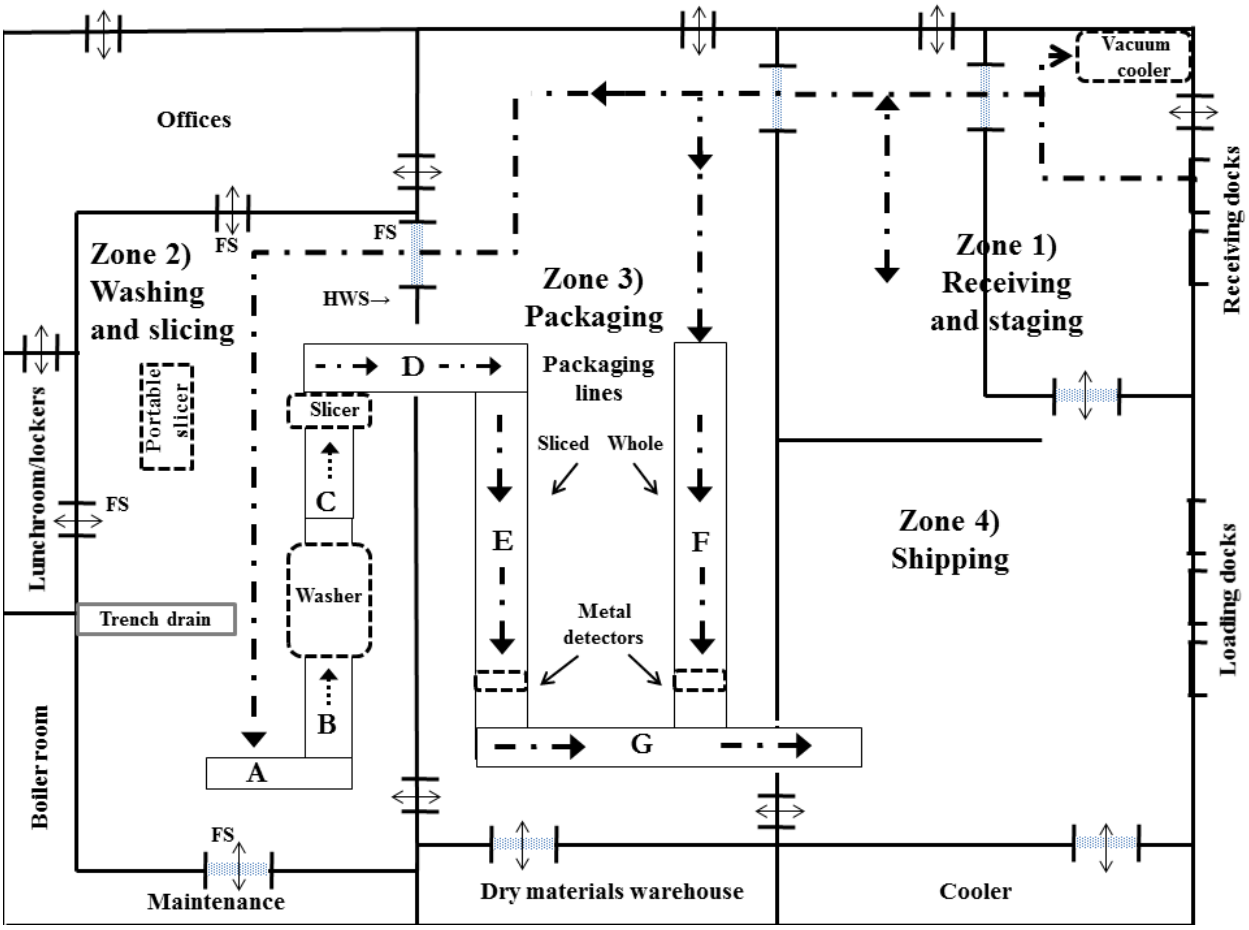

FIGURE 5: Mushroom processing facility layout and product flow. Letters A through F correspond to conveyor lines which are described in the text. Zone 5 areas include cooler, warehouse, offices, lunch and locker rooms. HWS - Hand washing station, FS - Floor sanitizer.

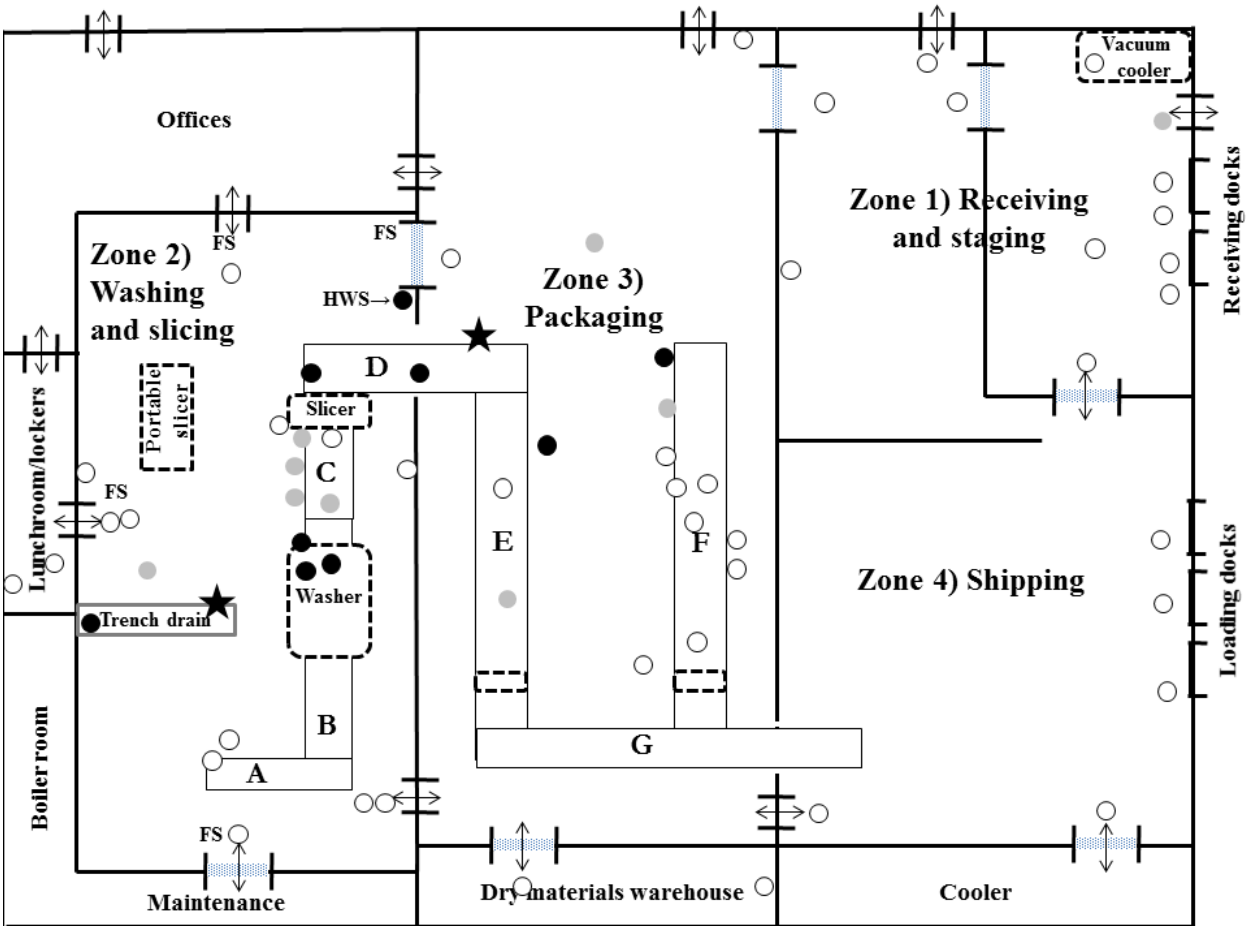

FIGURE 6: Distribution of *L. monocytogenes* in the mushroom processing facility. Each location was sampled during each of the 3 sampling periods. ○ = not detected at any period, ● = detected at one period, ● = detected at 2 periods, ★ = detected at all three sampling occasions.

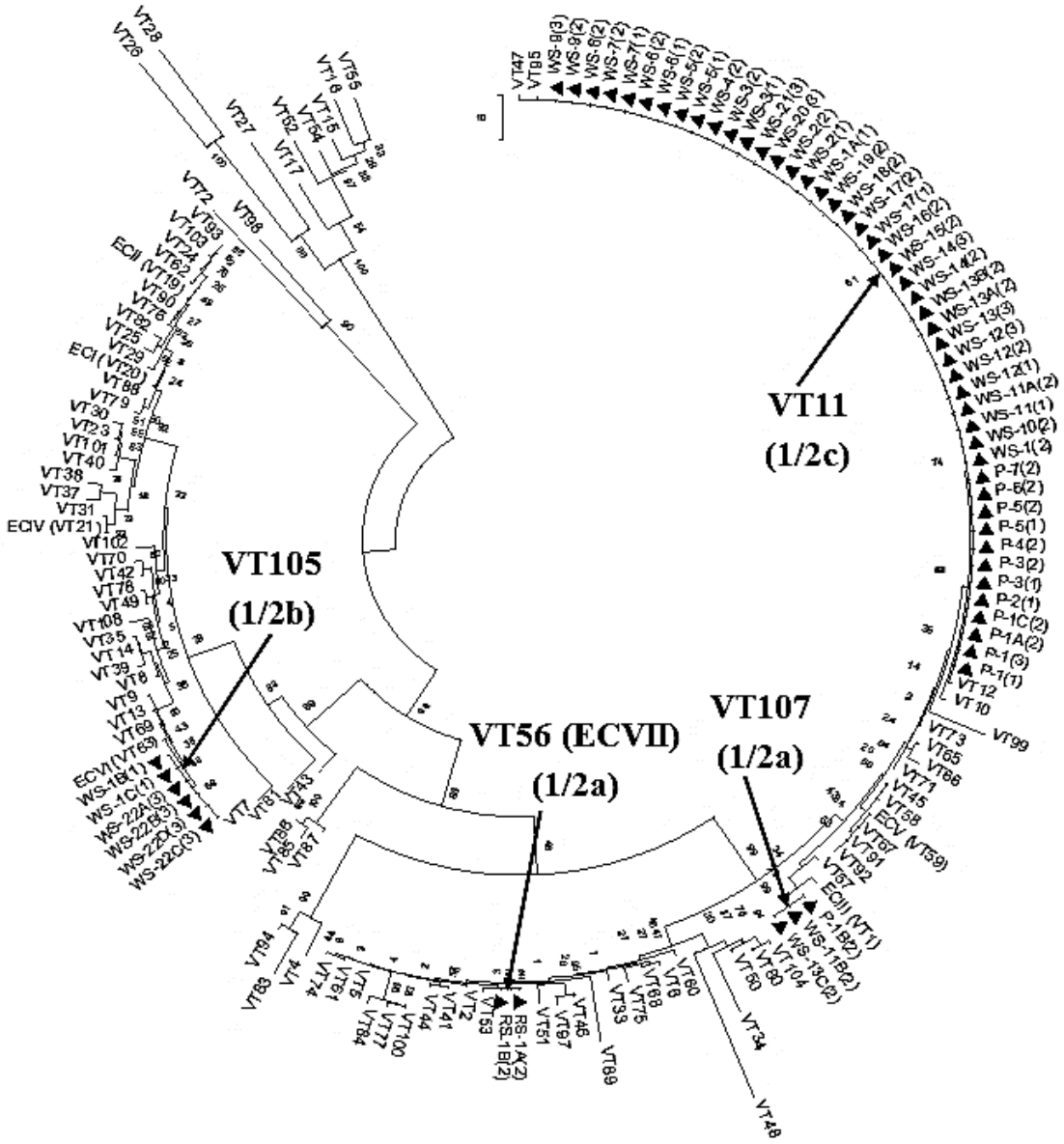

FIGURE 7: Phylogenetic relationship between *L. monocytogenes* isolates from the mushroom facility based on multi-virulence-locus sequence typing (MVLST). An unrooted Neighbor-joining tree algorithm with bootstrap values of 1000 replications are shown in the branches. *L. monocytogenes* isolates with filled triangles were from the mushroom processing facility with identification code: sample site (period of isolation). The other reference isolates were from author Knabel's MVLST database collection.

TABLE 2: Mushroom processing facility zones and sampling sites

Zone	Sampling period	No. of samples	Description of sampling sites
1) Receiving and staging	1 2 3	13 12 14	Loading dock doors, exit doors, floors, walls, vacuum cooler, wooden pallets, power switches, cardboard packaging boxes
2) Washing and slicing	1 2 3	30 43 43	Non-food contact surfaces on slicers, conveyers and washer, floors, floor mats, drains, walls, hand trucks, hand washing station, doors, door handles, cooler vents, overhead water pipeline, hoses, squeegees, electrical utility box
3) Packaging	1 2 3	19 23 23	Metal detectors, floors, non-contact surfaces on conveyers and weigh scales, inedible collection baskets, trays, doors, door handles, cooler vents, hand forklifts, electrical units, floor mats
4) Shipping	1 2 3	7 5 7	Dock floors, forklifts, loading truck, plastic curtains
5) Non - processing	1 2 3	4 5 7	Office and hallway floors, vending machine, floor scrubbers, warehouse and cooler floors, lunch room and locker room floors

TABLE 3: Prevalence and location of *Listeria* spp. in the mushroom processing facility

		***Listeria* spp. prevalence (n/N (%))**			
		Sampling period			
Zone	***Listeria* sp.**	1	**2**	**3**	**Total**
1) Receiving and staging	*L. monocytogenes*	0/13 (0)	1/12 (8.3)	0/14 (0)	1/39 (2.6)
	L. innocua	5/13 (38.5)	1/12 (8.3)	0/14 (0)	6/39 (16.7)
	L. grayi	0/13 (0)	0/12 (0)	0/14 (0)	0/39 (0)
2) Washing and slicing	*L. monocytogenes*	9/30 (30.0)	19/43 (44.2)	7/43 (16.3)	35/116 (30.2)
	L. innocua	0/30 (0)	1/43 (2.3)	3/43 (7.0)	4/116 (3.4)
	L. grayi	0/30 (0)	3/43 (7.0)	1/43 (2.3)	4/116 (3.4)
3) Packaging	*L. monocytogenes*	4/19 (21.1)	7/23 (30.4)	1/23 (4.3)	12/65 (18.5)
	L. innocua	0/19 (0)	0/23 (0)	0/23 (0)	0/65 (0)
	L. grayi	1/19 (5.3)	0/23 (0)	0/23 (0)	1/65 (1.5)
4) Shipping	*L. monocytogenes*	0/7 (0)	0/5 (0)	0/7 (0)	0/19 (0)
	L. innocua	0/7 (0)	0/5 (0)	1/7 (14.3)	1/19 (5.3)
	L. grayi	0/7 (0)	0/5 (0)	0/7 (0)	0/19 (0)
5) Non - processing	*L. monocytogenes*	0/4 (0)	0/5 (0)	0/7 (0)	0/16 (0)
	L. innocua	0/4 (0)	0/5 (0)	0/7 (0)	0/16 (0)
	L. grayi	0/4 (0)	0/5 (0)	0/7 (0)	0/16 (0)
Total n/N (%)	*L. monocytogenes*	13/73 (17.8)	27/88 (30.7)	8/94 (8.5)	48/255 (18.8)
	L. innocua	5/73 (6.8)	2/88 (2.3)	4/94 (4.3)	11/255 (4.3)
	L. grayi	1/73 (1.4)	3/88 (3.4)	1/94 (1.1)	5/255 (2.0)

n = samples positive for *Listeria* spp. N = Number of samples taken.

TABLE 4: Serotypes and virulence types of *Listeria monocytogenes* isolated from positive sites during each sampling period

Sampling zone and site description*		Sampling period		
		1	2	3
Zone 1: Receiving and staging				
RS-1	Floor crack at exit door	N.D.	1/2a (VT56)	N.D.
Zone 2: Washing and slicing				
WS-1	Equipment frame under conveyor B	1/2c (VT11), 1/2b (VT105)	1/2c (VT11)	N.D.
WS-2	Floor underneath conveyor B	1/2c (VT11)	1/2c (VT11)	N.D.
WS-3	Floor under junction conveyors B and C	1/2c (VT11)	1/2c (VT11)	N.D.
WS-4	Floor under disc slicer	N.S.	1/2c (VT11)	N.D.
WS-5	Metal frame under conveyor C	1/2c (VT11)	1/2c (VT11)	N.D.
WS-6	Floor under conveyor D	1/2c (VT11)	1/2c (VT11)	N.D.
WS-7	Floor crack at junction of conveyor D, E	1/2c (VT11)	1/2c (VT11)	N.D.
WS-8	Floor adjacent to portable slicer	N.S.	1/2c (VT11)	N.D.
WS-9	Floor mat near conveyor A	N.S.	1/2c (VT11)	1/2c (VT11)
WS-10	Floor in front of conveyor A	N.S.	1/2c (VT11)	N.D.
WS-11	Pool of water at end of trench drain	1/2c (VT11)	1/2c (VT11), 1/2a (VT107)	N.D.
WS-12	Floor crack at metal trench drain grate	1/2c (VT11)	1/2c (VT11)	1/2c (VT11)
WS-13	Floor crevice at trench drain	N.S.	1/2c (VT11), 1/2a (VT107)	1/2c (VT11)
WS-14	Floor in front of trench drain	N.S.	1/2c (VT11)	1/2c (VT11)
WS-15	Bottom step on stairs near conveyor B	N.S.	1/2c (VT11)	N.D.
WS-16	Floor in front of slicer	N.S.	1/2c (VT11)	N.D.
WS-17	Crevice under hand washing station	1/2c (VT11)	1/2c (VT11)	N.D.
WS-18	Handle on floor brush/squeegee	N.D.	1/2c (VT11)	N.D.
WS-19	Hand truck wheel	N.D.	1/2c (VT11)	N.D.
WS-20	Wheel on conveyor C	N.D.	N.D.	1/2c (VT11)
WS-21	Floor under entrance side of conveyor C	N.S.	N.S.	1/2c (VT11)
WS-22	Metal frame under conveyor C	N.D.	N.D.	1/2b (VT105)
Zone 3: Packaging				
P-1	Floor crack under conveyor D	1/2c (VT11)	1/2c (VT11), 1/2a (VT107)	1/2c (VT11)
P-2	Floor under end of conveyor E	1/2c (VT11)	N.D.	N.D.
P-3	Floor mat near conveyor E	1/2c (VT11)	1/2c (VT11)	N.D.
P-4	Floor under conveyor E metal detector	N.S.	1/2c (VT11)	N.D.
P-5	Floor crack under conveyor F	1/2c (VT11)	1/2c (VT11)	N.D.
P-6	Floor mat adjacent to conveyor F	N.D.	1/2c (VT11)	N.D.
P-7	Floor crack under cooler vent	N.D.	1/2c (VT11)	N.D.

**L. monocytogenes* was never detected in Zones 4 and 5. VT = virulence type. N.D. = no *L. monocytogenes* detected. N.S. = site not sampled.

Chapter 4

Quaternary ammonium compound tolerance and surface adherence of persistent and transient *Listeria monocytogenes* clones isolated from a mushroom processing facility

Running title: QAC tolerance and adherence of *Listeria monocytogenes* clones

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4.1 Abstract

A recent 13-month longitudinal survey from a commercial mushroom processing environment revealed four virulence types (VTs) of *Listeria monocytogenes*: VT11, VT107, VT105, and VT56, of which VT11 was predominant and persistent. Therefore, we compared differences between clones in terms of their tolerance to a quaternary ammonium compound (QAC) sanitizer and in their ability to firmly adhere to stainless steel (SS) and concrete coupons. QAC tolerance was determined for all clones grown for 20-h (peak cell density) and 7 days (long-term-survival (LTS) phase) at 35°C. The minimum inhibitory concentration values were 9.1 ppm for all clones, indicating no significant ($P > 0.05$) differences between persistent and transient clones. However, VT11 (0.61 to 0.65) and VT107 (0.62 to 0.65) grew to a significantly ($P \leq 0.05$) higher OD_{600} at sub-lethal QAC concentrations compared to VT105 (0.49 to 0.51) and VT56 (0.47 to 0.56). Inactivation kinetics at the in-use QAC concentration (200 ppm) revealed that the Weibull model produced a better fit of the data than the linear 1st order model. The Weibull model showed that LTS phase cells were 5.7 to 149.2 times more tolerant to 200 ppm QAC than the 20-h cells. Also, the predominant and persistent clone (VT11) was 2.6 to 220 times more tolerant to QAC than the other clones. No significant ($P > 0.05$) differences were observed among clones for the recovery of firmly adhered cells from concrete coupons. However, VT11 populations on SS coupons significantly ($P \leq 0.05$) decreased after 5 and 7 days suggesting an early dispersal from SS. Concrete coupons harbored significantly ($P \leq 0.05$) more cells (2.12 to 8.06 log CFU/cm^2) than SS (1.14 to 6.12 log CFU/cm^2) and were significantly ($P \leq 0.05$) more protective of *L.*

monocytogenes when treated with 200 ppm QAC. These results suggest that the observed predominance and persistence of VT11 may be due to its higher growth at sub-lethal QAC concentrations, higher QAC tolerance in the LTS phase, and a more rapid dispersal from surfaces thus allowing it to colonize new surfaces in the mushroom processing environment.

4.2 Introduction

Listeria monocytogenes is a Gram-positive, rod-shaped, facultatively anaerobic, non-spore-forming bacterium that causes listeriosis in humans. Relative to other pathogens, the incidence of listeriosis is low; nevertheless, the high mortality rate (~30%) makes it one of the most dangerous foodborne pathogens. *L. monocytogenes* serotypes 1/2a, 1/2b, and 4b account for 95% of human clinical cases (*33*) and serotype 1/2c has caused some sporadic illnesses. *L. monocytogenes* accounted for <1% of outbreaks associated with fresh produce until 2010 (*7*, *27*). However, the 2011 outbreak due to *L. monocytogenes* contamination of cantaloupes caused 147 illnesses and 33 deaths and is considered as one of the deadliest foodborne outbreaks in United States history (*27*, *48*). Since then, *L. monocytogenes* has caused multiple outbreaks involving novel food vehicles. Recent *L. monocytogenes* outbreaks associated with fresh produce consumption include stone fruits, caramel apples, and mung-bean sprouts in 2014 (*8*, *10*, *27*) and pre-cut salad in 2016 (*9*). In all of these fresh produce outbreaks, *L. monocytogenes* was traced back to the processing environment in which the foods were packaged (*9*, *27*), suggesting that the processing facility is the most likely source of *L. monocytogenes* contamination.

Fresh white button mushrooms (*Agaricus bisporus*) comprised 90% of the US mushroom market during 2014 - 2015 (*70*). The nutritional benefits of mushrooms have been recognized during the past decade, resulting in increased demand for mushroom production. While *L. monocytogenes* has not caused outbreaks associated with the consumption of fresh commercially grown *Agaricus* mushrooms, numerous recalls of

sliced mushrooms have recently occurred (*11*, *12*, *13*). Microbial surveys of commercially grown fresh produce also reported the presence of *Listeria* spp. on fresh mushrooms (*60*). A previous longitudinal survey from a commercial mushroom slicing and packaging environment showed that *L. monocytogenes* was detected in 18.8% of samples taken (*52*). Subtyping of *L. monocytogenes* using multi-virulence-locus sequence typing (MVLST) and serotyping revealed that Virulence Type 11 (VT11), serotype 1/2c, predominated and persisted in the processing environment at 2 sites for 13 months (*52*). Previous surveys from seafood (*35*), ready-to-eat deli meats (*61*), pork (*65*, *66*), and dairy (*30*) processing facilities also showed that certain subtypes of *L. monocytogenes* predominated and persisted within these processing facilities.

Routes of transmission of *L. monocytogenes* to finished products can include incoming raw materials or the processing environment, including equipment, employees, floors, and floor drains (*21*, *69*). Persistent subtypes from the processing environment have been implicated in several incidents of *L. monocytogenes* product contamination (*17*, *56*, *59*, *67*). In the previous longitudinal study (*52*), VT11 was isolated from the environment before the start of operation and after improvements were made to sanitation practices. This demonstrates the need to investigate whether VT11 has unique characteristics, compared to other *L. monocytogenes* clones found within this facility. Several hypotheses have been proposed to explain the persistence of specific subtypes in other food facilities, including improved adherence to surfaces and biofilm formation (*44*, *72*), higher tolerance to sanitizers and heavy metals (*51*), and resistance to phage attack

(*36*). Nevertheless, the factors responsible for causing the persistence of specific subtypes of *L. monocytogenes* are still unknown.

Differences in the ability of persistent and transient *L. monocytogenes* to grow in the presence of a sanitizer have been investigated by other researchers (*2*, *32*, *42*, *72*). However, these studies did not determine the differences in tolerance between persistent and transient *L. monocytogenes* at bactericidal or bacteriostatic concentrations of sanitizer. Persistence of *L. monocytogenes* has been defined as by Ferreira et al. (*21*) "the repeated isolation of *L. monocytogenes* strain(s) on different sampling days that has/have identical subtypes which is determined either by phenotypic or genotypic methods". However, in order to increase the chances of finding the persistent subtypes and identifying harborage sites, Murugesan et al. (*52*) redefined persistent subtypes as those that are isolated on all sampling occasions within certain locations in the facility. Persistence of *L. monocytogenes* is due to the growth and/or survival within certain sites in the facility. Finkel et al. (*22*) suggested that in order to survive and persist in the environment, bacteria enter into a dormant state where they are protected from several stresses. Previous studies have demonstrated that after extended incubation, *L. monocytogenes* cells enter a non-replicating minimal metabolic activity state termed the long-term-survival (LTS) phase (*29, 73, 75*). Other studies have used different terms for LTS phase including long-term-stationary phase for *Escherichia coli* K12 (*22*) and senescent phase for *Serratia* spp (*64*).

LTS phase cells of *L. monocytogenes* have been reported to be more tolerant to several stresses including high pressure processing and heat (*73*). Most fresh produce

processors rely on effective cleaning and sanitizing procedures as the main preventive control strategy for eliminating *L. monocytogenes.* Quaternary ammonium compounds (QACs) are a class of cationic compounds commonly used in food facilities for sanitizing purposes. QAC were reported to be more effective against Gram-positive bacteria compared to Gram-negative bacteria (*41*, *46*). QAC sanitizers disrupt bacterial cell wall and cause cytosolic leakage by binding to negatively charged phospholipids and proteins on the outer membrane. They also penetrate into the cell where they target carboxylic groups and cause cytoplasmic coagulation (*68*). However, to date, the sanitizer tolerance of LTS phase *L. monocytogenes* cells has not yet been studied.

Other widely investigated phenomena to possibly cause persistence of *L. monocytogenes* are adherence to surfaces and biofilm formation. Previous studies have used non-porous materials such as stainless steel coupons, polyvinyl chloride (PVC) microtiter plates, and glass slides for comparing surface adherence and potential to form biofilms between persistent and transient *L. monocytogenes* subtypes. Porous surfaces such as floors and floor drains are reported as major risk factors for *L. monocytogenes* contamination in food facilities (*3*, *4*, *21*, *67*, *69*). Additionally, the previous survey in the mushroom processing environment showed that VT11 was repeatedly isolated from 2 sites at 3 consecutive sampling periods: a trench drain in the washing and slicing area, and a concrete floor in the packaging area (*52*). These surfaces are generally made of cement and/or concrete, which is rough and porous, and have the potential to become harborage sites for *L. monocytogenes*. Nevertheless, there are no reports of these surfaces being tested to determine differences in populations of persistent and transient subtypes.

Therefore, the objectives of this study were: 1) to determine the effect of QAC on growth and survival of different *L. monocytogenes* clones isolated from the mushroom processing facility and 2) to evaluate variations in the recovery of firmly adhered cells from concrete and SS surfaces among persistent and transient *L. monocytogenes* clones.

4.3 Materials and methods

Bacterial culture preparation. Four *L. monocytogenes* clones obtained from the previous longitudinal survey of a commercial fresh mushroom processing environment (*52*) were used in this study: the predominant persistent clone, VT11 (serotype 1/2c), and three transient clones, VT107 (serotype 1/2a), VT105 (serotype 1/2b), and VT56 (serotype 1/2a). The isolates were stored at -20°C as 15% glycerol stock solutions prior to use. All *L. monocytogenes* isolates were grown in tryptic soy broth supplemented with 0.6% yeast extract (TSBYE) (BD Diagnostic Systems, Franklin Lakes, NJ, USA) for 20 h at 35°C followed by streaking on tryptic soy agar plates supplemented with 0.6% yeast extract (TSAYE) (BD Diagnostic Systems). One isolated colony from each *L. monocytogenes* clone was grown in individual test tubes containing 10 ml of TSBYE for 20-h (peak cell density after which the death phase was observed) or 7 days (LTS phase) at 35°C as previously described by Wen et al. (*73*).

Sanitizer preparation. Quorum Clear V, a quaternary ammonium compound (QAC) sanitizer from Ecolab, St. Paul, MN, USA, was used for all experiments. This QAC sanitizer was selected because it was used in the mushroom processing environment

for sanitizing purposes during the survey period. The active ingredients in the sanitizer are alkyl (C14, 50%; C12, 40%; C16, 10%) dimethyl benzyl ammonium chloride (3.0%), octyl decyl dimethyl ammonium chloride (2.25%), didecyl dimethyl ammonium chloride (1.35%), and dioctyl dimethyl ammonium chloride (0.90%). The in-use QAC concentrations for food-contact and non-food contact surfaces are 200 and 580 ppm, respectively. QAC was sterilized using a 0.22 µm filter (VWR International, Radnor, PA, USA) prior to use. All QAC concentrations were prepared in sterile deionized water and used within 15 min of preparation.

Minimum inhibitory concentration (MIC) assay. Minimum inhibitory concentrations (MIC) for each *L. monocytogenes* clone were determined using the microdilution broth assay. Briefly, 20-h cells were centrifuged at 12,000 rpm for 3 min at 20°C. The pellets were washed and re-suspended in appropriate amounts of phosphate buffered saline (PBS) solution to achieve OD_{600} values of 0.1 For each clone, cells were further diluted in PBS to achieve final concentrations of 10^4 CFU/ml. QAC concentrations ranging from 580 ppm to 0.6 ppm were prepared in 2X TSBYE. Fifty microliters of each QAC solution and 50 µl of *L. monocytogenes* cultures were added to a 96-well microtiter plate (Corning Inc., Corning, NY, USA) in duplicate wells. The plates were covered with the lid and gently tapped 3 times to mix the contents and then sealed with Parafilm® to minimize moisture loss during incubation. The microtiter plates were incubated at 35°C for 24 h and OD_{600} values were measured using a spectrophotometer (Multiskan™, Waltham, MA USA). Wells showing evidence of visual turbidity were

neutralized with 50 µl of neutralizing buffer (BD Diagnostic Systems) for 10 min and the contents were plated on TSAYE to confirm bacterial growth. *L. monocytogenes* cultures with no added QAC served as the control. Additionally, QAC concentrations with no added *L. monocytogenes* were included to ensure that QAC was free from contamination. MIC was not determined for LTS phase cells because the MIC assay cannot differentiate between QAC preventing germination or growth of LTS phase cells of *L. monocytogenes.*

Tolerance of *L. monocytogenes* to QAC. The effect of QAC on survival of different *L. monocytogenes* clones was determined at the in-use level and at concentrations lower than the in-use level, but higher than MIC levels. The concentration used on food-contact surfaces (200 ppm) was selected for the in-use QAC level because preliminary results showed a rapid die-off of *L. monocytogenes* cells at QAC levels used for non-food-contact surfaces (≥ 580 ppm). Five milliliters of 20-h or LTS phase cells of each *L. monocytogenes* clone was exposed to 200 ppm QAC for 0, 15, 30, 45, and 60 s at 20°C. At each time interval, 250 µl was aliquoted and mixed with 250 µl of neutralizing buffer (BD Diagnostic Systems). The mixture was neutralized for 10 min at room temperature and serially diluted in PBS, and then plated in duplicate on TSAYE plates. The plates were incubated at 35°C for 48 h.

To determine differences in tolerance between clones, seven concentrations between MIC and in-use levels were used: 9.1, 13.6, 18.1, 27.2, 36.3, 72.5, and 145.0 ppm. These low concentrations were chosen to simulate areas within the concrete floors of the mushroom processing facility that might have different concentration of QAC.

Briefly, 100 µl of different QAC concentrations was added to 900 µl of 20-h or LTS phase cells of each *L. monocytogenes* clone. The mixtures were then held at 35°C for 24 h. After exposure, samples were centrifuged at 12,000 rpm for 2 min at room temperature and the pellet was washed and re-suspended in 1 ml of PBS to remove QAC. Samples were serially diluted in PBS, and plated in duplicate on TSAYE plates. All TSAYE plates were incubated at 35°C and CFUs were enumerated after 48 h. The minimum bactericidal concentration (MBC) values for *L. monocytogenes* clones were calculated as the concentration that reduced the survival populations to below detectable limit of 1 log CFU/ml. *L. monocytogenes* cultures with no added QAC served as the control. Log reductions were calculated as the difference between control and QAC-treated samples.

Preparation of stainless steel and concrete coupons. Stainless steel (SS) (304 grade) and concrete coupons (each with 12.7 mm diam and 3.8 mm height), obtained from Biosurface Technologies, MN, USA, were used to determine adherence differences between *L. monocytogenes* clones. Briefly, SS coupons were soaked overnight in acetone at room temperature to remove any grease from the surface. After soaking, SS coupons were washed with 1% Alconox™ followed by rinsing in tap water and then deionized water. Concrete coupons were washed in a similar manner except that they were not soaked in acetone due to breakage of concrete fragments. The SS and concrete coupons were air dried overnight at room temperature and autoclaved before use.

Inoculation of coupons. For each *L. monocytogenes* clone, 20-h cells were centrifuged at 12,000 rpm for 3 min at 20°C. OD_{600} values were determined and adjusted to 0.1 using sterile PBS. One milliliter of each adjusted overnight culture was added to 1000 ml sterile beakers each containing 300 ml of TSBYE (~10^6 CFU/ml), ten SS and ten concrete coupons. This high inoculum level was chosen to minimize differences in growth rate between clones in the surrounding medium. After inoculation, 2 coupons of each surface type were immediately removed for day 0 enumeration. The beakers were then incubated at 30°C and coupons were removed on days 1, 2, 5, and 7 for plating and enumeration. The concrete coupon purchased from Biosurface Technologies is surrounded by a polycarbonate cup to hold the concrete in place. Therefore, in order to detect cells only from the concrete, the polycarbonate cup holding the concrete coupon in place was aseptically wiped with 70% ethanol.

Enumeration of firmly adhered *L. monocytogenes* cells from SS and concrete coupons. To remove any loosely adhered cells, the coupons were successively dipped 3 times in separate sterile tubes each containing 10 ml of PBS. Therefore, in this study, cells recovered from SS or concrete coupons after rinsing are termed "firmly adhered". The washed coupons were then placed in 10 ml PBS tubes each containing six borosilicate glass beads (6 mm diameter Pyrex, Corning Inc.) and vortexed for 1 min to completely disintegrate the concrete coupon and recover the maximum number of cells from the coupons. The contents were then serially diluted in PBS and plated in duplicate on TSAYE plates. The plates were then incubated at 35°C for 48 h and CFUs were

enumerated. Cells recovered from the surface were reported as log CFU/cm^2 based on the calculated surface area of the coupon. For all *L. monocytogenes* clones, two coupons for each surface material were enumerated on days 0, 1, 2, 5, and 7, and the experiment was replicated twice. In addition, planktonic cells from the surrounding growth medium (TSBYE) were also enumerated on TSAYE plates on corresponding sampling days.

QAC tolerance of firmly adhered *L. monocytogenes* cells from SS and concrete coupons. Based on results from the adherence assay, cells were grown on SS and concrete coupons for 1 and 7 days, and rinsed to remove loosely adhered cells as described above. After rinsing, the coupons were exposed to 5 ml of 200 ppm QAC for 1 min at room temperature and then transferred to another tube containing 5 ml of neutralizing buffer. Control (untreated) coupons were directly placed in 5 ml of neutralizing buffer after rinsing. Cells recovered from the coupons (log CFU/cm^2) were enumerated as described above in the enumeration section. Log reductions were calculated as the difference between cells recovered from control (no QAC) and treated (QAC exposed) coupons. On each sampling day, two coupons were sampled for both control and treatment, and the experiment was replicated twice.

Data analysis. For comparison of QAC tolerance between *L. monocytogenes* clones, the data obtained from the 200 ppm QAC inactivation study were modeled using the linear 1st order (1) and non-linear Weibull distribution (2) to calculate the best fit of the data (*71*).

$$\log\left(\frac{N}{No}\right) = -\frac{t}{D} \quad (1)$$

$$\log\left(\frac{N}{No}\right) = -\frac{1}{2.303}\left(\frac{t}{\propto}\right)^{n} \quad (2)$$

where N is CFU at time t, and N_0 is CFU at time 0, D is the decimal reduction time (time), α is the scale parameter (time), and n is the shape parameter. The scale parameter (α) value in the Weibull distribution describes the degree of tolerance to a stressful condition. A higher α value indicates increased tolerance by *L. monocytogenes* and vice versa. The shape parameter (n) describes the inactivation kinetics. For instance, n < 1 indicates that over time some cells had less probability of dying, n >1 indicates that some cells were increasingly susceptible to death, and n = 1 indicates that all cells were equally susceptible to death (*71*).

From the obtained fit, mean square error (MSE) was calculated for both linear and non-linear models to determine the best predictor of the data using the following equation.

$$Mean\ Square\ Error = \frac{\Sigma\ (predicted - observed)^2}{n} \quad (3)$$

where n is the number of observations. A model with the lowest MSE value indicates that the predicted values are similar to the observed values and suggests that it is the best fit model for the data.

Statistical Analysis. Significant differences between *L. monocytogenes* clones were determined using SAS statistical software (SAS Institute Inc., Cary, NC, USA). The

least significant difference method in ANOVA was used to determine clonal differences in QAC tolerance and surface adherence properties with an alpha value of 0.05.

4.4 Results

Determination of MIC values. OD_{600} values for each *L. monocytogenes* clone exposed to QAC concentrations between 0.6 and 580 ppm are shown in (FIGURE 8). The results revealed that all *L. monocytogenes* clones had a MIC value of 9.1 ppm. However, OD_{600} values were significantly ($P \leq 0.05$) higher for VT11 and VT107 at QAC concentrations below 9.1 ppm compared to VT105 and VT56 (FIGURE 8). Only OD_{600} values for VT11 at 0.6, 1.1, and 2.3 ppm were not significantly ($P > 0.05$) different than control (0 ppm) values. Although elevated OD_{600} values and visual turbidity occurred at 580 and 290 ppm, plating results demonstrated that *L. monocytogenes* growth occurred only at QAC concentrations below 9.1 ppm (data not shown).

Inactivation of 20-h and LTS phase *L. monocytogenes* cells at 200 ppm QAC. FIGURE 9a and 9b compare QAC inactivation kinetics for *L. monocytogenes* clones grown for 20-h and 7 days. MSE values for the Weibull model were lower than the 1st order linear model indicating that the Weibull distribution is the best predictor for QAC inactivation of *L. monocytogenes* cells (TABLE 5). This is also supported by shape parameter (n) values being greater or less than 1, signifying a non-linear relationship between QAC contact time and survivors (TABLE 5). This was also confirmed visually

in FIGURE 9a and 9b, which show that the Weibull model accurately predicted the observed values.

Non-linearity was more apparent in 20-h cells than in LTS phase cells. The 20-h cells showed a more rapid decrease in the first 15 s, followed by a more gradual decrease until 60 s (FIGURE 9a). Higher α values for LTS phase cells of all *L. monocytogenes* clones (1.60 - 14.70 s) compared to 20-h cells (0.01 - 2.20 s) indicate that the former cells were significantly ($P \leq 0.05$) more tolerant to QAC (TABLE 5). The ratio of α values between LTS phase and 20-h cells was lowest for VT11 (6.7) followed by VT105, VT107, and VT56 with 18.4, 56.4, and 160.0 values, respectively. The 20-h and LTS phase cells of the predominant and persistent clone (VT11) were significantly ($P \leq 0.05$) more QAC tolerant than the transient clones (VT107, VT105, and VT56) (TABLE 5).

Inactivation of 20-h and LTS phase *L. monocytogenes* at QAC concentrations between MIC and in-use levels. Log reduction values for 20-h and LTS phase cells for each *L. monocytogenes* clone after exposure to QAC at concentrations between 9.1 and 145 ppm are shown in TABLE 6. Control cell densities (no added QAC) for 20-h VT11, VT107, VT105, and VT56 were 8.49 ± 0.11, 8.16 ± 0.33, 7.86 ± 0.28, and 7.75 ± 0.29 log CFU/ml. Control cell densities for LTS phase VT11, VT107, VT105, and VT56 were 7.86 ± 0.02, 8.19 ± 0.14, 8.06 ± 0.04, and 7.55 ± 0.04 log CFU/ml, respectively (data not shown). No viable cells were detected at 72.5 ppm or 145 ppm and hence the MBC value for each clone was 72.5 ppm (TABLE 6). Five or greater log reductions of 20-h and LTS phase cells were achieved at QAC concentrations greater than or equal to 27.2 ppm.

However, viable cells were detected at QAC levels of 36.3 ppm or lower. Clonal differences in survival were only observed between LTS phase cells of VT56 and LTS phase cells of VT105, with the latter being more tolerant to QAC (TABLE 6) ($P \leq 0.05$).

Recovery of *L. monocytogenes* clones from SS and concrete coupons and the surrounding growth medium. FIGURE 10a and 10b show populations recovered from the SS and concrete coupons, respectively. Populations for each *L. monocytogenes* clone on SS and concrete increased significantly *(*$P \leq 0.05$) during the first 24 h of incubation. On SS coupons, VT11 and VT107 populations were significantly ($P \leq 0.05$) higher on day 2 compared to VT105 and VT56 (FIGURE 10a). However, VT11 and VT107 significantly ($P \leq 0.05$) decreased after day 2 from 5.98 and 6.12 to 4.74 and 5.26 log CFU/cm2 on day 7. VT56 populations decreased after day 1 from 5.99 to 5.19 on day 7 (FIGURE 10a). In contrast, VT105 populations constantly increased from 4.59 on day 1 to 5.36 log CFU/cm2 on day 7 (FIGURE 10a). Nevertheless, only VT11 was significantly ($P \leq 0.05$) lower on days 5 and 7 than the other clones.

Recovery of *L. monocytogenes* cells from concrete coupons was 0.69 to 2.60 log CFU/cm^2 higher compared to SS coupons (FIGURE 10b). In contrast to the results from SS coupons, none of the *L. monocytogenes* clones on concrete showed a significant ($P > 0.05$) decrease in populations after day 2. Although only VT11 cells decreased after day 5, populations were not significantly ($P > 0.05$) lower than the other clones (FIGURE 10b).

FIGURE 11 shows the populations of *L. monocytogenes* cells from the medium surrounding the coupons. Population levels for all *L. monocytogenes* clones increased in the first 24 h of incubation (FIGURE 11). Populations of VT11 and VT107 were significantly ($P \leq 0.05$) higher than VT56 and VT105 on day 2. No significant differences ($P > 0.05$) in cell population levels between clones were seen in planktonic cells after day 2 (FIGURE 11).

Recovery of firmly adhered *L. monocytogenes* cells from SS and concrete after treatment with 200 ppm QAC. TABLE 7 shows the log reductions of 1 and 7 day old firmly adhered *L. monocytogenes* cells on SS and concrete coupons treated with 200 ppm QAC. Similar to the results shown in FIGURE 10b, recovery of cells from concrete was higher than SS. However, for each *L. monocytogenes* clone, overall log reduction values for QAC-treated firmly adhered cells on concrete were significantly ($P \leq 0.05$) lower (0.08 to 0.63) than firmly adhered cells on SS (1.68 to 3.32) and planktonic cells from the surrounding medium (3.60 to 6.68) (TABLE 7). However, for VT11, log reductions for day 1 planktonic and firmly adhered cells on SS were not significantly ($P > 0.05$) different (TABLE 7).

4.5 Discussion

A previous 13-month longitudinal survey from a commercial mushroom processing environment (*52*) identified four *L. monocytogenes* clones using MVLST, of which, one clone (VT11) was predominant and persistent. Previous studies have

suggested that persistence of certain subtypes in the processing facility is due to adaptation to the environment in which they reside. This adaptation includes increased resistance or tolerance to sanitizers used and increased adherence to surfaces. Therefore, we evaluated whether VT11 had increased tolerance or resistance to QAC sanitizer and increased adherence to surfaces compared to other clones.

The results showed that the MIC value of QAC for all *L. monocytogenes* clones was 9.1 ppm. Previous studies have reported a MIC of QAC for *L. monocytogenes* ranging between 0.63 to 32 ppm (*1*, *43*, *49*, *68*). Persistent *L. monocytogenes* subtypes have been reported to possess higher MIC values than transient subtypes (*25*, *43*, *57*). However, in the present study, MIC values were not significantly ($P > 0.05$) different between persistent and transient clones (FIGURE 8). Nevertheless, higher OD_{600} values of VT11 at lower QAC concentrations suggest that VT11 may grow to a higher final cell density at sub-lethal QAC concentrations and therefore might possess a growth advantage over other clones. Fox et al. (*25*) also showed that below 4 ppm benzethonium chloride the persistent subtype isolated from a cheese production environment had increased growth compared to transient subtypes. Preliminary experiments conducted with 1% solutions of bovine serum albumin, glucose, and PBS showed that the turbidity at higher QAC concentrations was due to binding of QAC with proteins (data not shown).

Resistance to an anti-microbial compound has been defined as the ability of a bacterial strain to grow at concentrations that are bactericidal for other subtypes within a species (*6*). In the present study, none of the *L. monocytogenes* clones grew below 9.1 ppm (FIGURE 8) and therefore, they could not be considered QAC resistant. On the

contrary, previous studies have claimed that certain *L. monocytogenes* subtypes are resistant to QAC (*49, 51, 63*). However, in these studies, only non-bactericidal levels of 10 ppm or less were used.

Tolerance, on the other hand, is the ability of a strain to survive temporary exposure to concentrations above the MIC (*6*). In the present study, all clones exposed to QAC levels between 9.1 to 36.3 ppm for 24 h showed subsequent growth in nutrient media (TABLE 6) suggesting that all *L. monocytogenes* clones were tolerant to these QAC concentrations. The MBC value (72.5 ppm) (TABLE 6) obtained in the present study was higher than the MBC value (35 ppm) previously reported by Fazlara and Ekhtelat (*20*). Because reduction levels for all clones were lower for LTS phase cells than corresponding 20-h cells (TABLE 6), we can conclude that the transition to LTS phase causes the cells to become more tolerant to QAC. Higher tolerance of LTS phase cells compared to 20-h cells was also observed at the bactericidal concentration of 200 ppm for up to 60 s (FIGURE 9b).

Wen et al. (*73*) reported that LTS phase cells of *L. monocytogenes* were also significantly ($P \leq 0.05$) more tolerant to high pressure processing and heat, compared to cells in other phases. The higher tolerance of LTS phase cells was speculated to be the result of cytoplasmic condensation, which further decreased the water activity within the cell and thereby reduced the efficiency of the processing methods (*73*). During transition to LTS phase, a change in cell morphology from rod-shaped cells to coccoid cells was observed by Wen et al. (*73*). Modifications in gene expression have also been reported to occur during this transition (*74*). For instance, *treB, glpF-2,* and *dnaK* were upregulated

during the LTS phase of *L. monocytogenes* (*74*). *TreB* and *glpF-2* genes transport and accumulate compatible solutes, glycine betaine and trehalose within the cell and have been reported to assist in increased tolerance during cold and osmotic shock (*26, 34*). Studies have shown that *dnaK* acts as a molecular chaperone and offers protection against heat shock by preventing denaturation of essential proteins (*31*, *74*). Other genes involved in cell wall synthesis were also upregulated in the LTS phase cells (*74*).

In a recent study by Doan et al. (*18*), LTS phase cells of *L. monocytogenes* were shown to be more tolerant to the bactericidal concentration of antibiotics compared to cells in other phases. Such cells have historically been defined as persisters, which are tolerant to bactericidal antibiotic concentrations (*38*, *39*, *76*). Doan et al. (*18*) showed that 100% of LTS phase cells were antibiotic tolerant and therefore, the authors concluded that LTS phase cells of *L. monocytogenes* can be classified as persisters. Increased antibiotic tolerance of persister cells has been suggested to be the result of dormancy (*39*). Antibiotics are effective only against metabolically active cells. Therefore, when cells are in a dormant state with no cell-wall synthesis or translation activity, thus decreasing or preventing the action of antibiotics (*39*). Dormancy is induced by the production of the stress alarmone, guanosine tetraphosphate (ppGpp) that results in activation of toxin-antitoxin systems, which then produce toxins that inhibit cell growth (*37*, *39*, *76*). It is possible that a similar mechanism may explain the increased tolerance of LTS phase cells to QAC (observed in the current study) and other stresses (*73*). The current definition of persister cells is limited to antibiotic tolerance, the use of this term for QAC tolerant cells may not be warranted unless the definition is broadened to include

tolerance to other types of stresses. Additional research is needed to determine similarities and differences in mechanisms involved in QAC and antibiotic tolerance of LTS phase cells.

The higher QAC tolerance by LTS phase cells may partially be due to the non-replicating minimal metabolic activity state and/or the transcriptomic response which could prevent the action of QAC through reducing or preventing QAC penetration into the cell and/or inhibiting the coagulation of essential proteins. The results from this study do not establish a definitive link between QAC exposure and upregulation of genes associated with LTS phase; however, we speculate that the predominance and persistence of VT11 compared to the other clones found in the mushroom processing facility could be due to differences in the amount of expression of these LTS phase-associated genes. Factors such as the presence of efflux pumps, plasmids containing heavy metal resistance, *bcrABC* cassette, transposon *Tn6188*, *mdrL*, and some *qac* genes were reported to influence QAC tolerance in other *L. monocytogenes* subtypes (*19*, *47*, *49*, *51*, *57*). However, further research is needed to understand the mechanism behind the increased QAC tolerance of VT11.

The best fit of the QAC inactivation data to the non-linear Weibull model indicates that reduction rates change with treatment time. This change in reduction rates is more evident in 20-h old cells (FIGURE 9a) and can be explained by the presence of a mixed population of cells with different degrees of QAC tolerance. Differential tolerance by mixed population has also been reported by other authors when *L. monocytogenes* cells were exposed to pressure, temperature, desiccation, chlorine dioxide gas, and

antibiotics (*16, 24, 38, 45*). This phenomenon of mixed population has been well documented by Knudsen et al. (*38*) when treating 16-h *L. monocytogenes* cells with bactericidal concentration of norfloxacin. The authors demonstrated an initial rapid killing followed by a leveling off of population and concluded that 16-h old culture comprised of mixed population of non-persisters and persisters (*38*). Additionally, the authors showed a much slower destruction rate for 6 day old cells exposed to norfloxacin compared to 16-h old cells (*38*) suggesting that the older cells may contain a higher fraction of persisters.

The lower MSE values of the linear model for LTS phase cells compared to corresponding 20-h cells suggest that the LTS population consists of cells that are more uniformly tolerant to QAC (TABLE 6). Among the *L. monocytogenes* clones studied, both 20-h and LTS phase VT11 cells demonstrated higher tolerance to QAC compared to other clones (TABLE 5). The lower ratio of α between LTS phase and 20-h cells for VT11 suggests that the changes responsible for the transition to LTS phase may occur sooner in VT11 than in the other clones, which may explain why VT11 was able to persist in the mushroom processing facility.

Despite the greater tolerance of planktonic VT11 cells treated for 60 s at the "in-use" concentration of 200 ppm compared to other clones (TABLE 5), complete elimination of all clones, including VT11, was achieved for planktonic cells treated at 72.5 and 145 ppm for 24 h (TABLE 6). Although these concentrations are lower than the "in-use" concentrations used by the industry, harborage sites within the mushroom processing facility exposed to QAC levels below the critical limit of 72.5 ppm could

create a higher risk for *L. monocytogenes* contamination. Also, in practice, achieving adequate QAC exposure at these harborage sites may not be possible due to the constantly wet production environment that may dilute the QAC to very low concentrations.

In this study, the differences in firmly adhered cells on SS and concrete surfaces were compared between *L. monocytogenes* clones. Concrete coupons harbored more *L. monocytogenes* cells than SS (FIGURE 10). These results are in agreement with previous studies where rough and porous surfaces retained more cells than smooth and non-porous surfaces (*62*, *77*). The rough and porous concrete would provide an additional surface area for adherence and penetration of *L. monocytogenes* resulting in higher populations, compared to the smoother SS. It is likely that, during the rinsing method used in this study, planktonic cells entrapped within the concrete were not removed to the same extent as on SS. Therefore, we used the term firmly adhered cells to include cells that are both adhered to the sides of pores and cells that might be physical entrapped within pores.

Several other studies have shown that persistent *L. monocytogenes* subtypes are more adherent to SS and PVC microtiter plates than transient subtypes. (*5*, *44*, *54*, *72*). However, in the present study, VT105 (transient clone) increased in population over time on SS (FIGURE 10a). On the other hand, VT11 (predominant and persistent clone) populations on SS were lower than other clones on days 5 and 7 (FIGURE 10). This decrease cannot be explained by differences in populations in the surrounding medium because populations for all clones on day 5 and 7, including those for VT11, were not significantly ($P > 0.05$) different (FIGURE 11). Although the initial inoculum of all *L.*

monocytogenes clones was adjusted to the same OD_{600} prior to inoculation, cell counts for VT105 were lower than other clones suggesting that it might grow more slowly (FIGURE 11). Chae and Schraft (*14*) also showed variations in cell counts among *L. monocytogenes* subtypes with similar OD values.

The lower recovery of VT11 from SS on days 5 and 7 suggests that VT11 cells began to release or disperse from the surface sooner than cells from other clones (FIGURE 10a). Early dispersal from the surface can be triggered by several factors, including 1) depletion of nutrients, 2) scarcity of oxygen, 3) accumulation of toxic metabolites, 4) saturation of the material, 5) breakdown of the extracellular polymeric substances (EPS) layer for active colonization of new substrates, and 6) differential gene expression (*23*, *28*, *55*). VT11 may be more responsive to these changes than other clones, thus allowing it to rapidly disperse from surfaces during unfavorable conditions allowing it to colonize new sites throughout the mushroom processing facility.

The lower reduction of firmly adhered cells on SS and concrete, compared to planktonic cells after treatment with 200 ppm QAC (TABLE 7), might be due to 1) insufficient QAC contact due to limited diffusion through the multiple layers of cells or by the presence of EPS, 2) changes in cell physiology owing to the slower growth rate, and/or 3) differential gene expression between cells in the planktonic and firmly adhered states (*40*, *50*, *53*, *58*). The lower reduction was more evident in concrete (TABLE 7) due to its porous nature, which may further prevent bactericidal levels of QAC from reaching the cells present deep within pores. Chaitiemwong et al. (*15*) showed that deeper grooves on the SS surface prevented sanitizer from coming into contact with *L. monocytogenes*

cells and resulted in a lower reduction. It is likely that a concentration gradient of QAC is created within the concrete, which could result in QAC concentrations lower than MBC levels in areas where *L. monocytogenes* cells are deeply embedded. This hypothesis is also supported by the results obtained from the previous longitudinal survey of a commercial mushroom processing facility (*52*). The survey demonstrated that after implementation of intervention measures, VT11 was not detected in any of the samples taken before the start of the operation, but was detected from samples taken during operation (*52*), indicating that VT11 may be present deep within the concrete floors and move to the surface during operation.

Similar log reductions for day 1 VT11 planktonic and firmly adhered cells on SS (TABLE 7) suggest that adherence to SS did not offer added protection against QAC and indicate that mechanisms other than adherence are responsible for increased QAC tolerance by VT11. We speculate that the high inoculum concentration (10^6 CFU/ml) used in the adherence assay might have caused early transition of VT11 cells to LTS phase and thus resulted in overall higher tolerance. This is supported by the results of Wen et al. (*75*) who showed that *L. monocytogenes* cells entered the LTS phase within 24 h when using high initial inoculum levels. Additional research is required to determine the effect of longer term QAC exposure on firmly adhered *L. monocytogenes* cells within concrete and SS.

In conclusion, the predominant and persistent clone (VT11) had a higher growth at sub-lethal QAC concentrations, increased QAC tolerance in the LTS phase, and early dispersal of firmly adhered cells from surfaces. This combination of factors may provide

a growth and survival advantage for VT11, allowing it to predominate and persist over other clones in wet and porous floor and floor drain environments in the mushroom processing facility. This information can be used to develop novel strategies to eliminate persistent clones of *L. monocytogenes* from mushroom and other food processing environments. These strategies include 1) proper sealing of porous floors to prevent penetration of *L. monocytogenes*, 2) equipment sanitary design improvements that facilitate effective cleaning and sanitizing, and/or 3) improvements in facilities design and maintenance to prevent accumulation of water on floors, floor drains and equipment.

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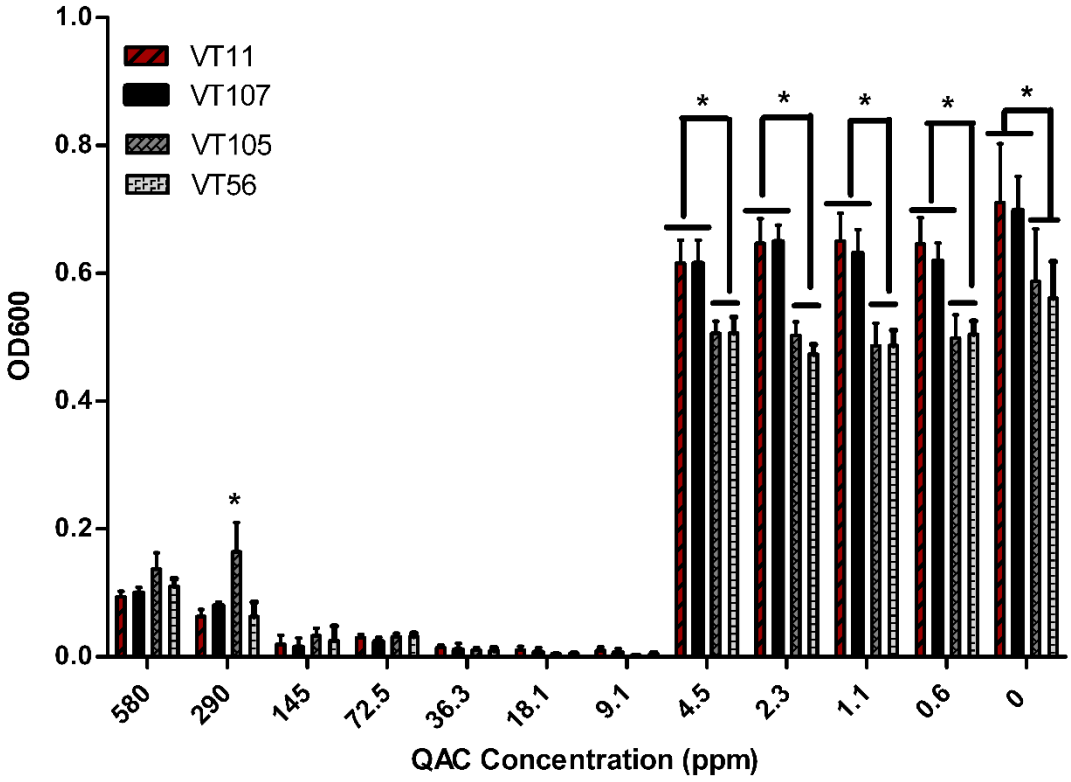

FIGURE 8: Minimum inhibitory concentration assay of *L. monocytogenes* clones after 24 h exposure to quaternary ammonium compound sanitizer at 35°C. * indicate significant differences between *L. monocytogenes* clones at a particular concentration ($P \leq 0.05$).

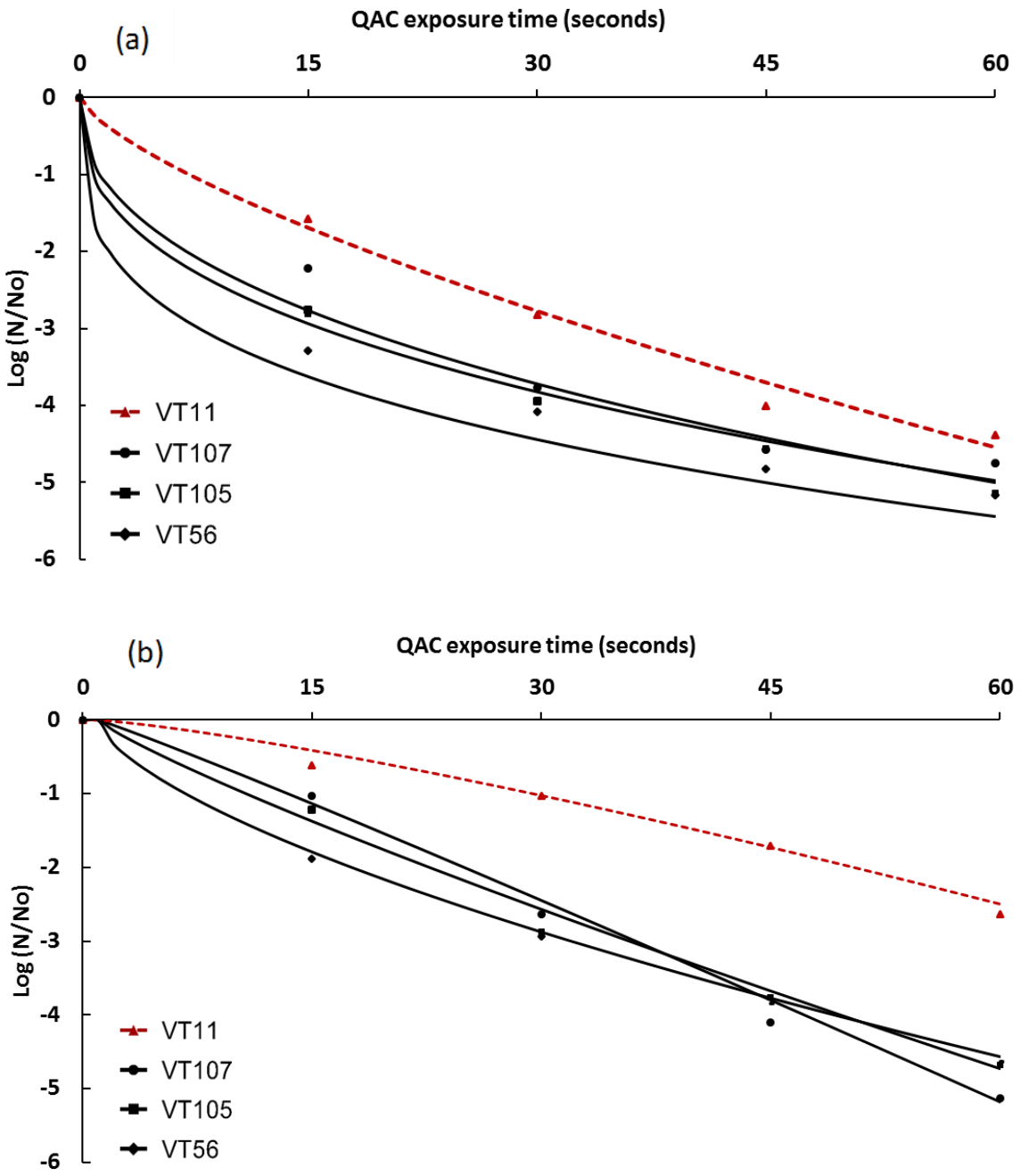

FIGURE 9: Tolerance of *L. monocytogenes* clones grown for 20-h (a) and 7 days (long-term-survival phase) (b) after exposure to 200 ppm quaternary ammonium compound (QAC) sanitizer at different time intervals at room temperature. The data were fitted using the non-linear Weibull distribution model.

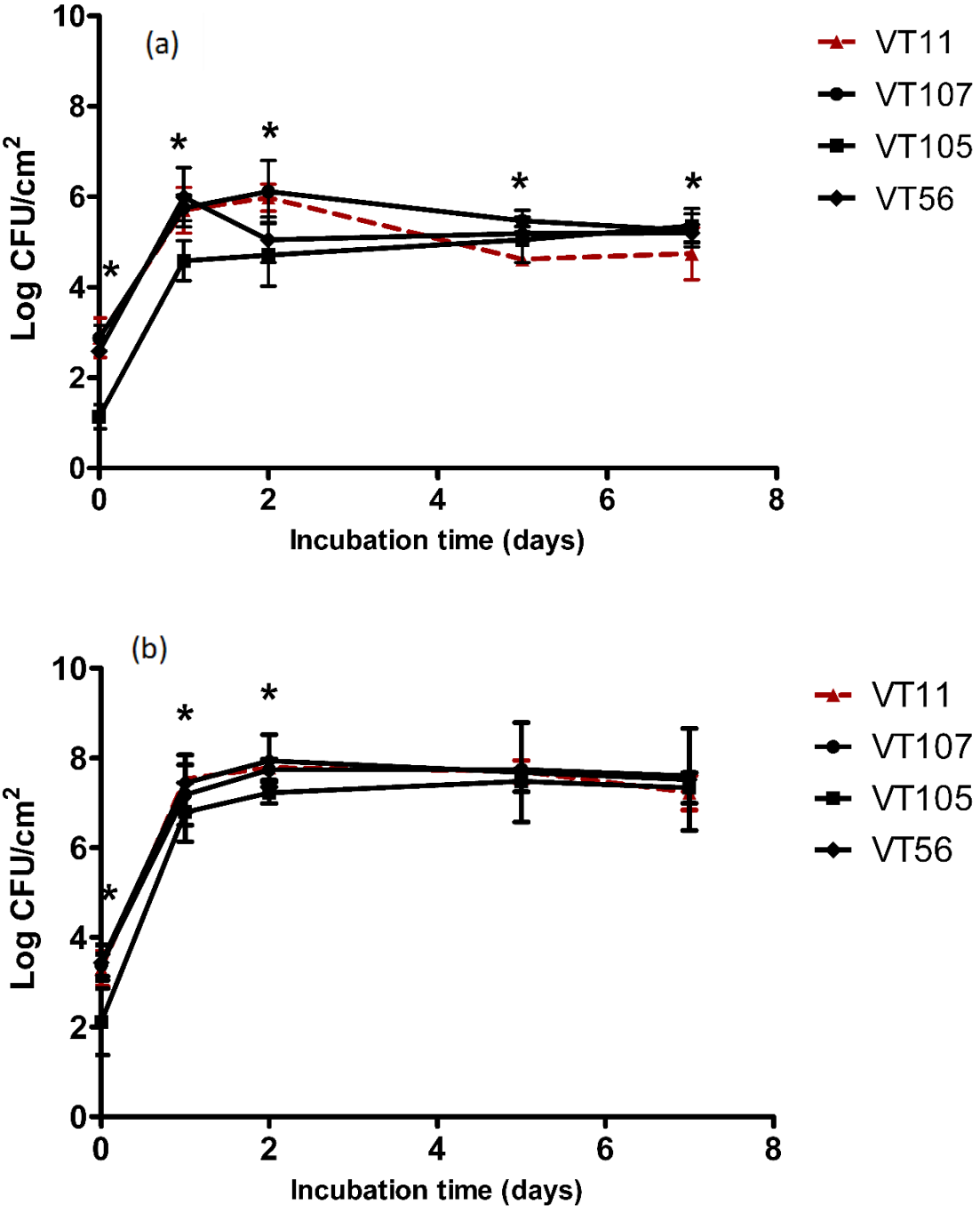

FIGURE 10: Recovery of *L. monocytogenes* cells from stainless steel (a) and concrete (b) coupons incubated in TSBYE at 30°C for 0, 1, 2, 5, and 7 days. * indicates significant differences between *L. monocytogenes* clones on any given day ($P \leq 0.05$).

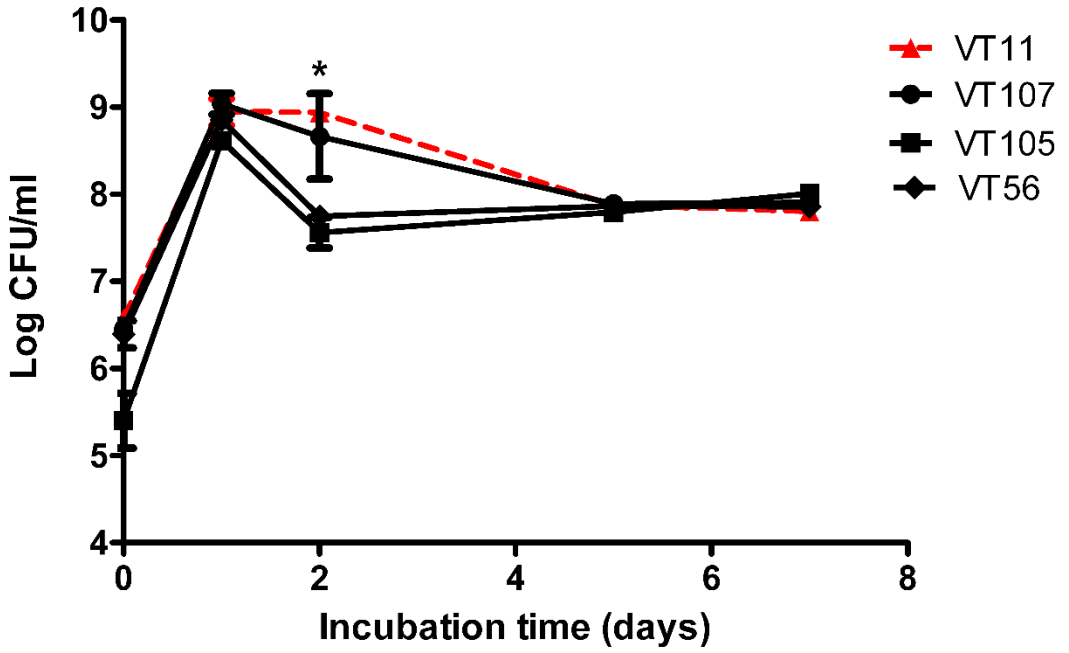

FIGURE 11: Planktonic cell populations in TSBYE medium surrounding SS and concrete coupons after 0, 1, 2, 5, and 7 days. * indicates significant differences between *L. monocytogenes* clones on any given day ($P \leq 0.05$).

TABLE 5. Mean square error (MSE) and Weibull model scale (α) and shape (n) parameter values for inactivation of planktonic cells of *L. monocytogenes* clones exposed to 200 ppm quaternary ammonium compound sanitizer at 20°C for up to 60 s.

Virulence type	20-h cells				Long-term-survival phase cells			
	MSE Linear	MSE Weibull	n	α (s)[1]	MSE linear	MSE Weibull	n	α (s)[1]
VT11	0.13	0.04	0.71	2.20^{aB}	0.02	0.01	1.26	14.70^{aA}
VT107	0.44	0.01	0.38	0.10^{bB}	0.02	0.02	1.06	5.64^{bA}
VT105	0.62	0.002	0.43	0.20^{bB}	0.04	0.02	0.86	3.67^{bA}
VT56	1.30	0.001	0.29	0.01^{bB}	0.15	0.00001	0.65	1.60^{bA}

[1]Each scale parameter value (α) represents the mean from 3 replicate experiments. Different lower case letters within a column and upper case letters within a row are significantly different ($P \leq 0.05$).

TABLE 6. Tolerance of planktonic 20-h and long-term-survival phase cells of *L. monocytogenes* clones exposed to different quaternary ammonium compound sanitizer concentrations at 35°C for 24 h.

Phase	Virulence type	Log reduction (CFU/ml)[1] QAC concentration (ppm) 9.1	13.1	18.1	27.2	36.3	72.5	145
20-h	VT11	2.79 ± 0.99[a]	3.82 ± 1.10[a]	5.00 ± 0.43[a]	6.63 ± 1.39[a]	6.83 ± 0.51[a]	> 7.49	> 7.49
	VT107	2.57 ± 0.89[a]	3.93 ± 1.61[a]	5.36 ± 1.24[a]	5.97 ± 1.09[a]	6.55 ± 0.93[a]	> 7.16	> 7.16
	VT105	2.72 ± 1.05[a]	3.78 ± 0.85[a]	5.27 ± 1.12[a]	6.19 ± 0.88[a]	6.86 ± 0.28[a]	> 6.86	> 6.86
	VT56	2.38 ± 0.99[a]	3.57 ± 0.93[a]	4.82 ± 1.39[a]	5.80 ± 0.86[a]	6.32 ± 0.58[a]	> 6.75	> 6.75
Long-term-survival	VT11	0.89 ± 0.66[ab]	2.28 ± 1.13[ab]	4.43 ± 0.75[ab]	6.21 ± 1.46[a]	6.55 ± 0.75[a]	> 6.86	> 6.86
	VT107	0.44 ± 0.35[b]	1.77 ± 1.02[ab]	3.30 ± 1.29[b]	6.75 ± 0.69[a]	6.56 ± 0.62[a]	> 7.19	> 7.19
	VT105	0.32 ± 0.19[b]	1.10 ± 0.28[b]	3.33 ± 0.97[b]	5.52 ± 0.27[a]	6.62 ± 0.74[a]	> 7.06	> 7.06
	VT56	1.80 ± 1.16[a]	3.20 ± 1.56[a]	6.06 ± 0.38[a]	6.47 ± 0.12[a]	6.64 ± 0.18[a]	> 6.55	> 6.55

[1]Each log reduction value were calculated as the difference between control (not exposed to QAC) and treated samples (exposed to QAC). Each data point represents mean and standard deviation from 3 replicates. Different letters within a column are significantly different ($P \leq 0.05$). Limit of detection for plating was 1 log CFU/ml.

TABLE 7. Tolerance of *L. monocytogenes* cells from stainless steel and concrete coupons and the surrounding medium after exposure to 200 ppm QAC for 1 min.

Virulence type		Log (CFU/cm^2 or ml)[1]					
		Day 1[2]			Day 7		
		Planktonic	Stainless steel	Concrete	Planktonic	Stainless steel	Concrete
VT11	Untreated	8.92 ± 0.09	5.83 ± 0.24	7.34 ± 0.55	7.69 ± 0.25	4.22 ± 0.23	8.43 ± 0.16
	Treated	5.32 ± 0.32	3.43 ± 0.29	6.90 ± 1.31	3.85 ± 0.68	1.68 ± 0.33	7.80 ± 0.85
	Reduction	3.60 ± 0.50^{bA}	2.40 ± 0.70^{aA}	0.57 ± 0.86^{aB}	4.33 ± 0.67^{aA}	2.55 ± 0.46^{aB}	0.63 ± 0.87^{aC}
VT107	Untreated	8.61 ± 0.24	5.37 ± 0.06	7.37 ± 0.21	8.25 ± 0.28	4.64 ± 0.11	8.15 ± 0.25
	Treated	2.80 ± 0.38	3.10 ± 0.14	7.12 ± 0.35	3.22 ± 1.00	2.33 ± 0.08	7.82 ± 0.33
	Reduction	5.80 ± 0.16^{aA}	2.27 ± 0.57^{aB}	0.26 ± 0.38^{aC}	5.26 ± 0.92^{aA}	2.31 ± 0.36^{aB}	0.33 ± 0.15^{aC}
VT105	Untreated	8.12 ± 0.18	4.76 ± 0.12	7.14 ± 0.19	8.17 ± 0.16	5.59 ± 0.27	7.65 ± 0.33
	Treated	2.82 ± 0.14	3.08 ± 0.12	6.84 ± 0.27	3.34 ± 0.82	2.59 ± 0.38	7.57 ± 0.39
	Reduction	5.31 ± 0.37^{aA}	1.68 ± 0.16^{aB}	0.30 ± 0.18^{aC}	4.98 ± 0.83^{aA}	3.00 ± 0.55^{aB}	0.08 ± 0.37^{aC}
VT56	Untreated	8.73 ± 0.15	5.75 ± 0.03	7.56 ± 0.01	7.99 ± 0.01	4.95 ± 0.30	8.45 ± 0.14
	Treated	2.05 ± 0.86	2.43 ± 0.19	7.44 ± 0.15	3.77 ± 1.46	2.39 ± 0.25	7.94 ± 0.67
	Reduction	6.68 ± 0.87^{aA}	3.32 ± 0.13^{aB}	0.11 ± 0.16^{aC}	5.79 ± 0.92^{aA}	2.57 ± 0.56^{aB}	0.50 ± 0.70^{aC}

[1]Log reductions were calculated as the difference between untreated (not exposed to QAC) and treated coupons (exposed to 200 ppm QAC. Each data point represents mean and standard deviation from 2 replicates. Different lower case letters within a column and upper case letters within a row are significantly different ($P \leq 0.05$).
[2]No significant differences were observed in log reduction values between days ($P \leq 0.05$) and therefore they are not shown in the table.

Chapter 5

Growth and peak cell densities of persistent and transient *Listeria monocytogenes* clones in mushroom broth and a model study to explain the persistence of a predominant *L. monocytogenes* clone in a commercial mushroom processing facility

Running title: Growth of *L. monocytogenes* in mushroom nutrients

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5.1 Abstract

Four *Listeria monocytogenes* virulence types, VT11, VT107, VT105, and VT56, isolated from a previous 13- month longitudinal survey of a commercial fresh-cut mushroom processing facility were evaluated for their differences in growth rate and peak cell density (PCD) in fresh mushroom broths (MBs). To provide possible explanations of persistent *L. monocytogenes* contamination within the mushroom processing facility, the effect of surface sanitization with 580 ppm QAC on *L. monocytogenes* cells was determined using concrete and stainless steel (SS) coupons. Five different fresh MB dilution levels, 30%, 10%, 5%, 0.5% and 0.05%, were used to grow *L. monocytogenes* clones at 35°C for 10 days and 10°C for 30 days. The results showed that all *L. monocytogenes* clones were able to grow on all MB dilutions at 35°C. However, at 10°C, 0.05% MB only supported the survival, but not growth, of *L. monocytogenes* clones. The generation time of *L. monocytogenes* clones in MB dilutions ranged between 38.45 and 207.06 min at 35°C and from 9.27 to 28.42 h at 10°C. VT11 grew faster to a higher PCD at 0.5% MB and 0.05% MB at 35°C, but significantly ($P \leq 0.05$) higher at 10°C indicating a growth and survival advantage over other clones in a nutrient-limited environment. Surface sanitization with 580 ppm QAC for 10 min did not kill *L. monocytogenes* cells present deep within concrete coupon, thus allowing a survival population of 3.60 log CFU/cm^2; however, complete elimination of *L. monocytogenes* cells was achieved for SS coupons. Based on the results from the present and previous chapters, a model was proposed to explain the persistence of a predominant *L. monocytogenes* clone in the previously surveyed mushroom processing facility.

5.2 Introduction

The demand for whole and fresh-cut produce has increased in recent years due to convenience and recognition of health benefits associated with consumption of fruits and vegetables. However, there are growing concerns in the fresh produce industry about the increasing number of outbreaks and recalls caused by contamination with foodborne pathogens. Fresh commercially grown mushrooms occupy 90% of the US mushroom market during 2014 - 2015 (*51*). Mushrooms have not been associated with any foodborne outbreaks to date; however, several recalls of sliced mushrooms contaminated with *Listeria monocytogenes* have occurred recently in the United States and Canada (*4, 5, 6, 7, 8, 53*).

A microbial survey of retail fresh produce in Washington state reported that 1.0% of fresh mushrooms were contaminated with *L. monocytogenes*, 5.0% with *Salmonella*, and 4.0% with enterohemorrhagic *Escherichia coli* (*42*). An earlier study by Heisick et al. (*17*) in the Minneapolis area reported that 12.0% of mushrooms were positive for *L. innocua*; however, *L. monocytogenes* was not isolated from any of the mushrooms. Studies have shown that *L. monocytogenes* is capable of growing on whole and sliced *Agaricus* mushrooms (*11, 15, 25, 26*). Sliced mushrooms provide the most favorable environment for the growth of *L. monocytogenes* due to: 1) increased release of intracellular nutrients, 2) increased presence of water, and 3) increased surface area for attachment (*11, 25*). *L. monocytogenes* is a strict intracellular pathogen that is capable of surviving and growing within mammalian host cells. Immunocompromised people, including pregnant women, neonates, children, and elderly, with depressed T- cell mediated immunity are much more susceptible to listeriosis than healthy populations (*20,*

55). Due to its high mortality rate (~30%), *L. monocytogenes* is a major concern for food processors, public health officials, and consumers.

An earlier 13- month longitudinal survey of a commercial mushroom slicing and packaging environment conducted by our group reported the prevalence of *L. monocytogenes* to be 18.8%, primarily from the wet processing floors (*36*). Subtyping of these isolates, identified using multi-virulence-locus sequence typing (MVLST), revealed 4 different virulence types: VT11 (serotype 1/2c), VT107 (serotype 1/2a), VT105 (serotype 1/2b), and VT56 (serotype 1/2a). Of these clones, VT11 was predominant in the processing environment and was persistent at two sampling sites. Studies from seafood, ready-to-eat deli meats, pork, and dairy processing facilities have also reported predominance and persistence of specific subtypes of *L. monocytogenes* (*16, 21, 44, 47, 49*). Persistent subtypes have been shown to form dense biofilms (*29, 57*), tolerate higher concentrations of sanitizers (*13, 27, 37*), or possess heavy-metal resistance (*35*). However, factors causing persistence of *L. monocytogenes* are not yet completely understood.

Persistence of *L. monocytogenes* is achieved by growth and/or survival in a given environment. *L. monocytogenes* is always in competition for nutrients with other organisms in natural habitats and food processing environments; therefore, it is necessary for *L. monocytogenes* to utilize available nutrients in an effective way for its growth and/or survival. However, limited information is available regarding the differences in growth rate and survival of *L. monocytogenes* in a food matrix from which the persistent and transient subtypes were originally isolated. This is important because differential

utilization of nutrients in a competitive environment may offer a growth and/or survival advantage for persistent subtypes over transient subtypes.

Because *L. monocytogenes* is a ubiquitous organism in many natural habitats, its introduction into production and/or processing facilities through raw materials, employees, equipment, and farm animals is inevitable. However, effective cleaning and sanitizing practices have been shown to reduce *L. monocytogenes* contamination in food facilities (*21*, *22*, *23*, *28*, *45*). Quaternary ammonium compound (QAC) sanitizers are commonly used on food-contact and non-food-contact surfaces in food facilities to control *L. monocytogenes*. QAC adsorbs to the bacterial cell surface, diffuses through the cell wall, and binds to the cytoplasmic membrane. This binding causes disruption of cytoplasmic membrane thus releasing the cytoplasmic constituents and causing cell death (*34*).

Chaitiemwong et al. (*9*) showed that surface sanitizing was not sufficient to destroy *L. monocytogenes* cells present within the deeper grooves of the stainless steel (SS) coupon due to limited contact with *L. monocytogenes* cells. However, the effect of surface sanitizing to destroy *L. monocytogenes* cells that are deeply embedded within a rough and porous concrete surface has not yet been studied. This is important because porous floors and floor drains in food processing facilities have been reported as harborage sites for *L. monocytogenes* (*1*, *2*, *12*, *48*, *49*). Additionally, the previous longitudinal survey we conducted identified a trench drain in the washing and slicing area, and a concrete floor in the packaging area as harborage sites for the predominant and persistent *L. monocytogenes* clone, VT11 (*36*).

Therefore, the objectives of this study were (1) to determine the growth rate and peak cell density (PCD) of *L. monocytogenes* clones at different dilution levels of mushroom broth, (2) to evaluate the effect of QAC surface sanitization on survival of *L. monocytogenes* on both external and internal surfaces of the concrete and SS coupons, and 3) to propose a model explaining the predominant and persistent VT11 contamination in the commercial mushroom processing facility.

5.3 Materials and methods

Bacterial culture preparation. Four *L. monocytogenes* clones obtained from the previous longitudinal survey of a commercial mushroom processing environment (*36*) were used in this study: VT11 (serotype 1/2c, predominant and persistent clone), and VT107 (serotype 1/2a), VT105 (serotype 1/2b), and VT56 (serotype 1/2a) (all transient clones). Before each experiment, frozen cultures of *L. monocytogenes* were revived in 10 ml tryptic soy broth supplemented with 0.6% yeast extract (TSBYE) (BD Diagnostic Systems, Franklin Lakes, NJ, USA) for 20 h at 35°C with subsequent streaking on tryptic soy agar plates supplemented with 0.6% yeast extract (TSAYE) (BD Diagnostic Systems).

Mushroom broth preparation. Fresh whole white button mushrooms (*A. bisporous*) were purchased from local grocery stores one day prior to the experiment. Differences in growth rates and PCDs among *L. monocytogenes* clones were evaluated at 30, 10, 5, 0.5 and 0.05% fresh mushroom broth (MB) dilution levels. These mushroom

concentrations were chosen to represent areas within the porous concrete floors in the mushroom processing environment. A 30% stock MB was made by grinding 30 g fresh mushrooms and 70 g phosphate buffered saline (PBS) solution for 1 min (Osterizer, Oster, Shelton, CT). The 30% MB was further diluted in appropriate amounts of PBS for preparation of other MB dilutions.

Inoculation of mushroom broth. For each *L. monocytogenes* clone, one isolated colony was grown in 10 ml TSBYE for 20 h at 35°C. The cultures were centrifuged at 12,000 rpm for 3 min at 20°C, and the pellets were washed and re-suspended in PBS to contain 10^5 CFU/ml. One ml of the diluted culture was added to 100 ± 1 g of MB respectively, to achieve a final concentration of ~10^3 CFU/ml. MBs inoculated with individual *L. monocytogenes* cultures were then incubated at 35°C for 10 days or 10°C for 30 days. Incubation at 10°C was included in this study to simulate the temperature of the washing and slicing area in the mushroom processing facility (*36*).

Aliquots were taken at appropriate time intervals and were serially diluted with PBS. The samples were then plated in duplicate on oxford agar plates supplemented with modified anti-microbic supplement (MOX) (BD Diagnostic Systems). The plates were incubated at 35°C for 48 h before enumeration. *Listeria*-like colonies were visually identified as grayish white colonies with zones of blackening. The limit of detection for plating was 1 log CFU/ml. The experiment was repeated three times at both temperatures for each MB dilution level. *L. monocytogenes* cultures inoculated into TSBYE were used as controls. Additionally, 30% MB with no added *L. monocytogenes* was used to ensure that the mushrooms did not contain any *Listeria* spp. that may interfere with the results.

Generation time and peak cell density calculation. Generation times (min or h) of *L. monocytogenes* clones were calculated using the following equations (1 & 2).

$$\mu = \frac{2.3 \log N/No}{\Delta t} \quad (1)$$

$$t_d = \frac{0.693}{\mu} \quad (2)$$

where t_d is the generation time, μ is the growth rate, N is CFU at time t, N_0 is CFU at time 0, and Δt is the time interval.

Peak cell densities (PCD) were calculated by averaging time points after the peak cell density was reached. At 35°C, for all clones, PCD was calculated as the average of log CFU/ml after 20 h. At 10°C, PCD values were calculated as the average of log CFU/ml of all time points after 9 days for 30% and 10% Mushroom broth (MB) dilutions; 12 days for TSBYE and 5% MB; and 3 days for 0.5% and 0.05% MB dilution levels. The time intervals for PCD calculations at 35°C and 10°C were selected based on the results of the growth curve experiment.

Preparation of stainless steel and concrete coupons. Reduction of *L. monocytogenes* using QAC surface sanitization was determined using SS (304 grade) and concrete coupons obtained from Biosurface Technologies (Bozeman, MT, USA). Coupon dimensions were 12.7 mm diam X 3.8 mm height. SS coupons were soaked overnight in acetone at room temperature to remove any grease and then washed with 1% Alconox™ followed by rinsing with tap water and deionized water. Concrete coupons were washed in a similar manner except that they were not soaked in acetone due to this solvent

causing extensive breakage into fragments. The washed coupons were air dried overnight at room temperature and were autoclaved before use. To simulate a continuously wet surface, concrete coupons were soaked for 16 h in fresh sterile TSBYE and air dried for 15 min in a sterile laminar flow cabinet prior to inoculation with *L. monocytogenes.*

Sanitizer preparation. Quorum Clear V (Ecolab, St. Paul, MN, USA), a QAC sanitizer**,** was selected for this study because it was used for sanitizing both food-contact (200 ppm) and non-food-contact surfaces (580 ppm) in the previously surveyed mushroom processing facility. QAC was sterilized using a 0.22 µm filter (VWR International, Radnor, PA, USA) before use. The results from chapter 4 demonstrated that the QAC concentration of 200 ppm used for food contact surface was not effective in killing firmly adhered *L. monocytogenes* cells within concrete. Therefore, for the purpose of this study, the manufacturer's recommended QAC concentration for non-food-contact surfaces (580 ppm) was used. This level was used in the mushroom processing facility for sanitizing floors and floor drains. QAC was prepared in sterile deionized water and used within 15 min of preparation.

QAC treatment of stainless steel and concrete coupons. TSBYE soaked sterile concrete and SS coupons were inoculated with 20 µl of 20-h old VT11 cells. The results from chapter 4 revealed that VT11 (the predominant persistent clone) was significantly more tolerant to QAC compared to other clones and therefore, this clone was selected to determine the effect of surface sanitization. The inoculated coupons were placed in a sterile petri dish. Sterile Whatmann filter paper pieces were placed inside the petri dish

and rehydrated with 1 ml of sterile deionized water. The petri dish was then sealed with Parafilm® to minimize dehydration of the inoculum. The coupons were incubated at 30°C for 24 h (1st incubation).

After incubation, the coupons were successively dipped 3 times in separate sterile tubes each containing 10 ml of PBS to remove any loosely adhered cells. The rinsed coupons were then covered with 30 ml of 580 ppm QAC solution and held for 10 min at room temperature. Two separate sterile swabs were used for removing cells from the surface of the coupons. After each treatment, the exterior surface of the coupons was swabbed 3 times each in both clockwise and counter clockwise directions. The swabs were then placed in tubes containing 5 ml neutralizing buffer (BD Diagnostic systems) and six 6-mm borosilicate glass beads for 10 min at room temperature. The surface swabbed coupons were added to separate test tubes each containing 5 ml of neutralizing buffer and glass beads. The swabs and coupons were vortexed for 1 min to dislodge *L. monocytogenes* cells. After vortexing, aliquots obtained from the vortexed coupons and swabs were plated on TSAYE in duplicates and incubated at 35°C for 48 h and then enumerated. Coupons that were rinsed in PBS but not treated with QAC were served as controls. Cells from the external and internal surfaces of the control coupons were enumerated in the same manner as treated coupons. The limit of detection of *L. monocytogenes* cells from the coupons was 0.6 log CFU/cm^2.

Additionally, to determine the effect of adding fresh media to survival populations of *L. monocytogenes* within concrete, 20 µl of fresh TSBYE was added to the surface of another set of treated and control SS and concrete coupons. The coupons were further incubated at 30°C for 24 h (2nd incubation) and enumerated as described above.

Cement slab construction for research model. To determine whether sanitizer and mushroom nutrients can penetrate into porous floor surfaces, a model cement slab was constructed in the lab using Rapid Set Cement All® (Home Depot, State College, PA, USA). Four parts of the cement were mixed with 1 part deionized water according to the manufacturer's instructions. 25 g of the blend was immediately poured into ice cube trays and allowed to set at room temperature for 24 h. After setting, the cubes were removed from the tray and placed in deionized water for 3 days for curing. At the end of the curing period, the pH of the cement slab was 7.21. The dimensions of the cement slab were 45 mm in length, 24 mm in width, and 17 mm in height. The cement slab was autoclaved prior to use.

Riboflavin penetration assay. Riboflavin (Sigma Aldrich, St. Louis, MO, USA) was used to simulate the penetration of sanitizer or mushroom nutrients into a porous concrete or cement floor and floor drain. Riboflavin, a water-soluble dye, was selected over direct measurement of sanitizer or mushroom nutrients because it can fluoresce under UV light at 365 nm and thus, the differences in color (concentration) intensities can be visually observed. Riboflavin has previously been used as a tool to detect leaks in food processing equipment valves and to determine the efficiency of cleaning for clean-in-place processes (*41*, *58*). Briefly, 100 µl of 200 ppm riboflavin solution was added to the surface of the cement slab. The riboflavin was allowed to penetrate for 2 or 24 h at room temperature. After penetration, the cement slab was broken using a hammer and the top and side of the concrete fragment were viewed under the UV light. The depth of

penetration was measured using a Vernier caliper. The riboflavin penetration assay was not performed on the concrete coupons from Biosurface Technologies because they were not able to sustain the force during breaking.

Statistical analysis. SAS statistical software (SAS Institute Inc., Cary, NC, USA) was used to calculate differences in growth rates and PCDs between *L. monocytogenes* clones. Least significant difference method of ANOVA was used for all comparisons between *L. monocytogenes* clones and MB dilution levels with an α value of 0.05.

5.4 Results

Growth rate of *L. monocytogenes* clones at 35°C and 10°C. TABLE 8 shows that all *L. monocytogenes* clones were able to grow at all MB dilution levels at 35°C. However, at 10°C, *L. monocytogenes* clones did not grow in 0.05% MB, except for one of the replicates of VT11 (TABLE 8). A biphasic or diauxic growth was observed at 30% (VT105 and VT56) and 10% (all 4 clones) MB concentrations (FIGURE 12). However, growth of *L. monocytogenes* was minimal in the second growth phase of the biphasic curve and therefore, it was not included in the growth rate calculation. The growth rate of *L. monocytogenes* clones ranged between 38.67 and 208.25 min at 35°C, and from 9.38 and 28.58 h at 10°C (TABLE 8).

Growth rates for all *L. monocytogenes* clones were significantly ($P \leq 0.05$) lower in 0.05% MB compared to other MB dilutions and TSBYE at 35°C (TABLE *8*). In addition, at 35°C, a lag time of approximately 5 h was observed for all *L. monocytogenes*

clones in 0.05% MB (FIGURE 12). No significant ($P > 0.05$) differences in growth rate were observed among *L. monocytogenes* clones in 30%, 10%, 5% and 0.05% MB dilutions. However, in 0.5% MB at 35°C, VT11 and VT105 had significantly ($P \leq 0.05$) higher growth rate compared to VT107, but not significantly ($P > 0.05$) different than VT56 (TABLE 8).

At 10°C, for all *L. monocytogenes* clones, growth rates in MB dilutions were similar to TSBYE. No significant ($P \leq 0.05$) differences in growth rates were observed between *L. monocytogenes* clones in all MB dilutions and TSBYE at 10°C (TABLE 8). However, VT56 had significantly lower growth rate in 0.5% compared to other MB dilutions (30%, 10%, and 5%) and TSBYE (TABLE 8). Although VT11 had higher growth rate in 0.5% MB at 10°C, it was not significantly ($P > 0.05$) different from other clones (TABLE 8).

Peak cell densities of *L. monocytogenes* clones at 35°C and 10°C. PCD of *L. monocytogenes* were significantly ($P \leq 0.05$) affected by MB dilution and temperature (TABLE 9; FIGURE 12). In general, for all *L. monocytogenes* clones, TSBYE supported a significantly ($P \leq 0.05$) higher PCD than other MB dilutions (TABLE 9). At both temperatures, PCDs of all *L. monocytogenes* clones were significantly ($P \leq 0.05$) higher in 5% MB followed by 10%, 30%, 0.5%, and 0.05% MB dilutions (TABLE 9). The highest PCD of *L. monocytogenes* clones in 5% MB dilution ranged between 7.14 and 7.28 log CFU/ml at 35°C and from 6.23 to 6.55 log CFU/ml at 10°C (TABLE 9).

For all *L. monocytogenes* clones, at 35°C, PCDs started to decline after 35 h in 0.5% MB (undetected on 240 h) and 25 h in 0.05% MB (undetected after 148 h)

(FIGURE 12). However, at 10°C, PCDs for all clones rapidly decreased after 3 days and were below detectable limit after 15 days (except for VT105 which was not detected after 9 days) (FIGURE 12). Nevertheless, one of the VT11 replicates survived for up to 21 days at 10°C (data not shown).

PCDs of all *L. monocytogenes* clones in 5% and 10% MB dilutions were significantly ($P \leq 0.05$) higher at 35°C compared to 10°C (TABLE 9). However, for all *L. monocytogenes* clones, significant ($P > 0.05$) differences in PCDs were not observed between 35°C and 10°C in 30% (except VT56) and 0.5% MB dilutions (TABLE 9).

Significant ($P \leq 0.05$) differences in PCDs among clones were mainly observed at 10°C. VT105 had significantly lower PCDs compared to VT11 at all MB dilution levels (TABLE 9; FIGURE 12). However, at 10°C, VT11 and VT107 had significantly ($P \leq 0.05$) higher PCD than VT105 and VT56 at 0.5% MB dilutions (TABLE 9).

Effect of surface sanitization on *L. monocytogenes* cells. TABLE 10 shows cell populations recovered from SS and concrete after exposure to 580 ppm QAC for 10 min at room temperature. After treatment, VT11 cells were not detected on the exterior surface of SS and concrete coupons (TABLE 10). However, 3.60 and 2.69 log CFU/cm^2 were recovered from the interior surface of the concrete coupon during 1st and 2nd incubation (TABLE 10). Nevertheless, *L. monocytogenes* cells present within concrete did not grow after addition of fresh TSBYE prior to 2nd incubation.

Riboflavin penetration into the model cement slab. Since the riboflavin penetration results after 2 and 24 h were the same, only 2 h data are shown in FIGURE

13. Riboflavin penetrated into the model cement slab to a distance of 11 mm (65% of the total slab height) after 2 h. A visual difference in riboflavin color intensity was observed within the model cement slab (FIGURE 13). The riboflavin fluorescence was higher at the external surface (top) of the model cement slab compared to the internal surface (within) of the model cement slab (FIGURE 13).

5.5 Discussion

The results from a previous 13- month longitudinal survey of a commercial mushroom processing facility revealed that a single clone of *L. monocytogenes* (VT11) was predominant and persistent on the wet processing floors where water and mushroom residues were abundant (*36*). Therefore, we evaluated differences in growth rate and PCD between *L. monocytogenes* clones at different MB dilution levels to simulate the porous concrete floor in the mushroom processing environment. The results showed that all *L. monocytogenes* clones were able to grow and/or survive in each of the MB dilution levels (TABLE 8; FIGURE 12).

All *L. monocytogenes* clones had significantly ($P \leq 0.05$) lower PCDs in MB dilutions compared to the nutrient dense TSBYE medium. 100 mL of TSBYE contains 1.7 g tryptone, 0.3 g soytone, and 0.25 g glucose and can support higher PCD. On the other hand, Mattila et al. (*33*) reported that 100 g of fresh white button mushrooms (*A. bisporous*) contain 92.3 g moisture and 7.7 g dry matter; however, the maximum amount of mushrooms used in this study was 30 g. The 7.7 g dry matter consists of 4.5 g carbohydrates (1.5 g dietary fiber), 2.1 g protein, 0.33 g crude fat, and 0.78 g ash (*33*);

The major carbohydrates present in white button mushrooms are chitin, glucose, mannitol, glycogen, and trehalose (*10*, *33*, *54*). Of these carbohydrates, chitin and glucose comprise 1.5 g (*33*) and 1.48 g (*52*), respectively. *L. monocytogenes* can utilize various carbohydrate molecules including glucose, glycerol, fructose, mannose, maltose, rhamnose, and chitin (*24*, *50*). The biphasic growth shown in FIGURE 12 suggests that *L. monocytogenes* can utilize more than one carbohydrate source from mushrooms. Preliminary results showed that all *L. monocytogenes* clones did not hydrolyze synthetic chitin (Sigma Aldrich) (data not shown). We speculate that *L. monocytogenes* will utilize glucose in MBs more rapidly for increased ATP synthesis followed by utilization of other carbohydrates resulting in a biphasic growth.

Mushrooms also contain 17 of 20 essential amino acids including cysteine, methionine, valine, isoleucine, and leucine (*33*) which have been reported as essential for the growth of *L. monocytogenes* (*43*, *50*). Other micronutrients found in mushrooms that have been shown to promote the growth of *L. monocytogenes* include riboflavin, thiamine, and niacin, (*32*, *50*). Since growth occurred in all MB dilution levels, it is apparent that mushrooms provide an excellent growth medium for *L. monocytogenes* (TABLE 8 and TABLE 9). This is in agreement with previous studies which showed that *L. monocytogenes* can reach a PCD of 5 to 9.5 log CFU/g on fresh mushrooms (*11*, *15*, *25*, *26*). The growth and survival of *L. monocytogenes* at 0.05% MB (0.004 g dry matter) are concerning because even very low concentrations of mushrooms can allow *L. monocytogenes* to grow.

The growth and survival of all *L. monocytogenes* clones indicate that they are adapted to the nutrients in mushrooms (FIGURE 12). Verghese et al. (*56*) also

demonstrated that the type of food-conditioning-film (FCF) can affect growth and biofilm formation of epidemic clones (ECs) of *L. monocytogenes*. The authors reported that ECs isolated from meat processing facilities formed denser biofilms on meat-conditioning films compared to other FCFs (*56*). They concluded that these ECs may have adapted to the food matrix from which they were originally isolated, resulting in increased growth and biofilm formation on FCFs (*56*).

The higher PCDs of VT11 and VT107 at the two lowest MB concentration levels (0.5% and 0.05% MB) at 35°C and 10°C suggest that these two clones might be better adapted to mushroom nutrient-limited environment compared to other clones. Nevertheless, VT11 was the only clone with both a high growth rate and high PCD at low MB concentration (TABLE 8; TABLE 9). Previous studies have similarly shown that persistent subtypes can grow more rapidly than non-persistent subtypes under acidic and salt stresses (*30*, *40*). In the present study, the low MB dilution levels would represent a nutrient-limited environment posing a starvation stress on *L. monocytogenes*. Growth and survival in a nutrient-limited environment might offer a competitive advantage for VT11 over other transient clones. Over time, these slight differences between clones in low nutrient environmental conditions could lead to more pronounced differences over time, and thus may explain the predominance and persistence of VT11 in wet floors and drain areas in the mushroom processing facility. Future research is needed to understand whether VT11 and VT107 use any alternate metabolic pathways that allow them to survive at very low nutrient levels.

Interestingly, for all *L. monocytogenes* clones, a lower PCD was observed at the 30% MB dilution level compared to 10% and 5% MB dilutions. Preliminary experiments

showed that *L. monocytogenes* growth rates and PCD values were higher in 5% filter-sterilized fresh MB compared to non-sterilized 5% fresh MB (Appendix A). Previous studies have reported that certain indigenous microorganisms present in processing facilities can inhibit the growth and survival of *L. monocytogenes* (*3*, *18*, *59*). Although we did not characterize microbial population levels on mushrooms, our preliminary results showed that the initial total plate counts on mushrooms were 6.14 log CFU/g (data not shown). Competition with background microorganisms for nutrients, oxygen, and/or space might explain the lower PCD observed in 30% MB (TABLE 9). The presence of natural toxic compounds in mushrooms, such as chitin, and/or production of inhibitory compounds by background organisms might also contribute to the lower PCD at 30% MB (TABLE 9). In contrast, significantly ($P \leq 0.05$) higher PCD at 5% MB might be due to lower amounts of background organisms for competition, resulting in increased availability of mushroom nutrients, and/or presence of less toxic compounds.

Differences in growth rate and PCD between *L. monocytogenes* clones were more evident during incubation at low temperature (10°C) (TABLE 8 and TABLE 9). At low temperatures, bacterial cell membranes typically become rigid causing reduced transport of molecules in and out of the cell. However, *L. monocytogenes* which is a psychrotroph overcomes this membrane rigidity by modifying the composition of fatty acids in the cell wall. These modifications include an increase in the ratio of C15:0 to C17:0 fatty acids, increased production of unsaturated fatty acids, and an increase in the ratio of ante-iso to iso- fatty acids (*14*, *46*). These changes decrease interactions between adjacent carbon molecules, thus increasing membrane fluidity at low temperatures (*14*). The lower PCDs

of *L. monocytogenes* at 10°C may be due to lower overall metabolism or expenditure of energy molecules for these membrane modifications rather than growth.

The surface sanitization study clearly demonstrated that QAC was less effective in destroying VT11 cells present deep within the porous concrete surface, compared to the smooth SS surface (TABLE 10). Lower QAC effectiveness on concrete coupons might be due to cells that are deeply embedded being exposed to lower QAC concentrations (below minimum bactericidal concentration). The riboflavin penetration study supports these two hypotheses by revealing a difference in color intensities between the external and internal surfaces of the model cement slab and suggests that a gradient of sanitizer concentration may occur within porous concrete floors (FIGURE 13). Martinez-Suarez (*31*) also proposed dilution of sanitizer in the processing environment, or biodegradation can result in a gradient of sanitizer concentration. Thus, areas with inadequate or improper sanitization may allow the formation of harborage sites that protect *L. monocytogenes* against destruction by QAC.

The results coincide with our hypothesis for lower log reduction on concrete coupons from chapter 4 where *L. monocytogenes* cells might be deeply embedded within the porous concrete coupon where sanitizer may not reach and kill the cells. The absence of growth of *L. monocytogenes* during the 2nd incubation on both exterior and interior surfaces of the concrete coupons (TABLE 10) might be due to the addition or penetration of low amounts of TSBYE after the 1st incubation. This low amount of TSBYE may be adequate for survival but not growth of *L. monocytogenes*. Paiva et al. (*38*) also demonstrated that *L. monocytogenes* can penetrate into a concrete surface. The authors showed that the application of BioSeal sealant before and after inoculation can help

reduce the survival populations of *L. monocytogenes* by preventing their penetration into concrete; however, it cannot eliminate *L. monocytogenes* completely from the concrete slab. Therefore, additional research is needed to understand whether sealing of concrete floors in the mushroom processing facility with polyester or epoxy sealants would prevent *L. monocytogenes*, mushroom nutrients, or water from penetrating into the porous floors and thus, prevent the formation of harborage sites.

On the other hand, Tompkin (*49*) suggested that scrubbing floors and floor drains with caustic soda followed by rinsing, application of sanitizer, and air drying is adequate to control *L. monocytogenes* contamination. Jessen and Lammert (*19*) also suggested that scrubbing floors was the most effective cleaning method to eliminate *L. monocytogenes*. However, possible structural damages (cracks and crevices) caused by these treatments must be taken into consideration when implementing these control strategies.

Based on the collective results from chapters 4 and 5, we propose a model (FIGURE 14) that may explain how the predominant VT11 clone may have persisted in the mushroom processing facility. Before the start of the mushroom processing operations, VT11 may be present both on the surface and deeply embedded within the rough and porous concrete floors. Routine cleaning and sanitizing procedures can kill VT11 on the floor surface, but not necessarily those cells present deep within the concrete (TABLE 10). It is also possible that shorter sanitization time or improper application of sanitizer might further limit the amount of sanitizer reaching deeply embedded cells.

Over time, the sanitizer may penetrate into the porous floors; however, water used for cleaning and sanitizing purposes as well as juices that exude from mushrooms during

processing may also penetrate into the porous concrete floors. Penetrating water and/or mushroom juices may dilute the sanitizer to levels well below the recommended concentration and possibly to minimum bactericidal concentration levels, thus creating a gradient of sanitizer concentration (in-use to 0 ppm) within the porous concrete floors. Areas within the concrete floor where sanitizer cannot penetrate could then act as harborage sites for *L. monocytogenes*, in particular VT11. This phenomenon of harborage sites has been well documented on food-contact equipment such as slicers and dicers (*28, 39*). The results from chapter 4 revealed that LTS phase VT11 cells were more tolerant to in-use QAC concentration and thus could have a survival advantage over other clones (FIGURE 9; TABLE 6). The results from chapter 4 also demonstrated that, at sub-lethal QAC concentrations, VT11 can grow to a higher PCD levels, thus resulting in a long term growth advantage over other clones (FIGURE 8).

Apart from sanitizer dilution, mushroom nutrients may also become diluted within the porous floors, resulting in a nutrient-limited environment. The results from this chapter (TABLE 9) revealed that VT11 can grow rapidly to a higher PCD at lower mushroom nutrient levels and transition to LTS phase sooner resulting in both growth and survival advantage over other clones. Early transition to the LTS phase may offer added protection against other stresses such as cold, oxidative, and osmotic stresses. During commercial operations, water and mushroom nutrients are reintroduced into the processing environment which may further dilute the sanitizer, thus lowering the sanitizer stress and allowing VT11 to multiply within the harborage sites. Thus, VT11 is more likely to form biofilms within these harborage sites compared to other clones. The results from chapter 4 indicated that, over time, firmly adhered VT11 can disperse sooner

(FIGURE 10) and thus recontamination of floor surfaces and colonization of new surfaces may be favored within the mushroom processing environment. This cycle is repeated daily, thus allowing VT11 to predominate and persist in the mushroom processing facility.

This model is also supported by the results of the previous longitudinal survey (*36*) where VT11 was not detected before the start of the operation (after application of QAC salts on the floor surface), but was detected during operation. This observation indicates that VT11 can move out of the concrete floor during operation and re-contaminate the floor surface. A limitation of this model is that it cannot explain how VT11 and other *L. monocytogenes* clones were originally introduced into this mushroom processing facility.

In conclusion, mushrooms provide an excellent growth environment for all *L. monocytogenes* clones tested in this study. However, VT11 grows rapidly to a higher PCD at low MB concentration levels compared to other clones, that may offer a growth and survival advantage over other clones. The model proposed might help us understand the complex factors that provide a competitive advantage for VT11 in the mushroom processing facility. This model may also find applications for explaining the previous well-known observations associated with persistent contamination of *L. monocytogenes* in other food processing facilities. If true, this model could be used in developing control strategies to eliminate *L. monocytogenes* from mushroom and other food processing facilities.

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5.6 References

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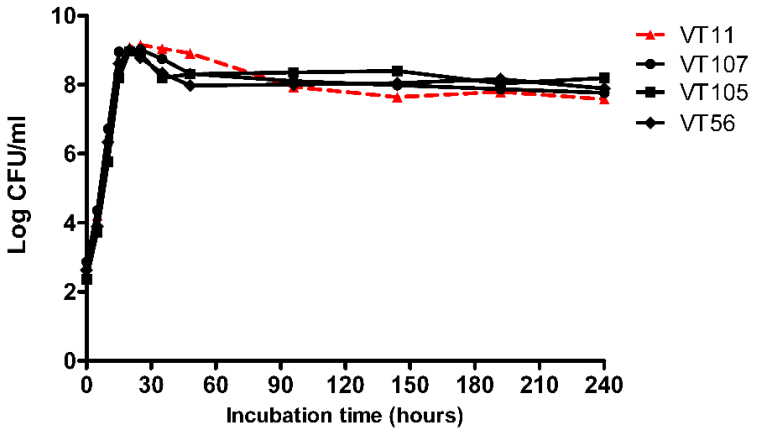
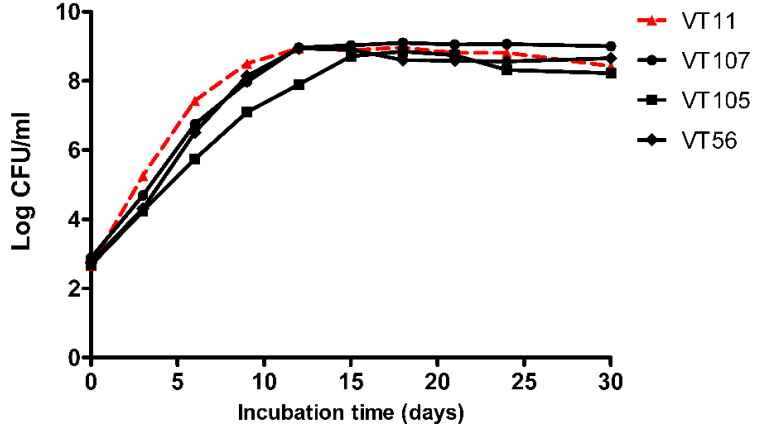
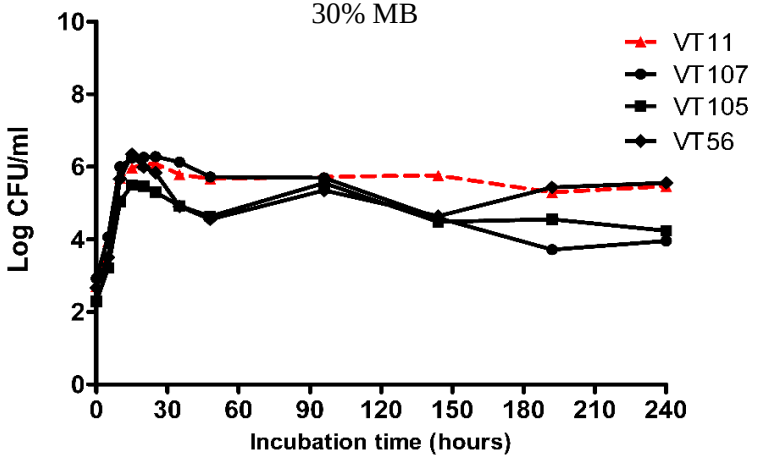
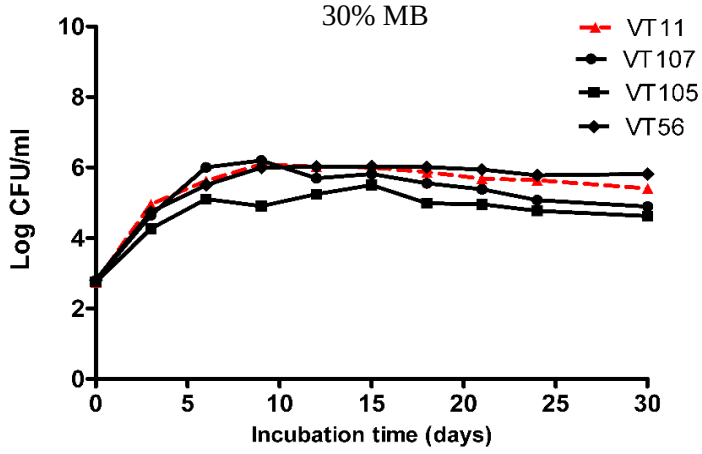
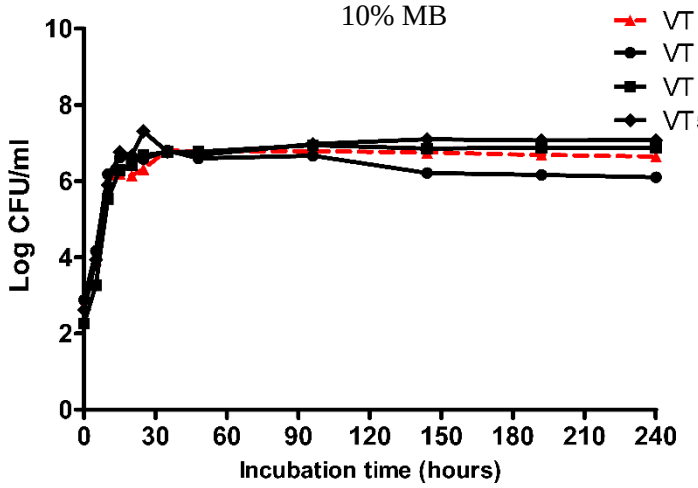
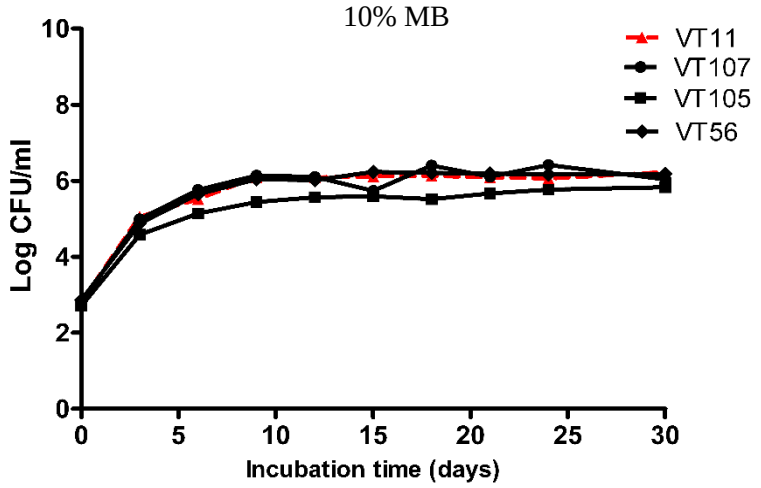
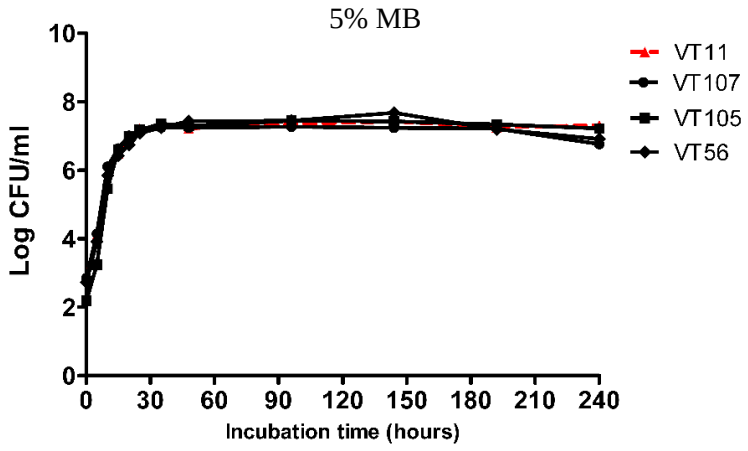
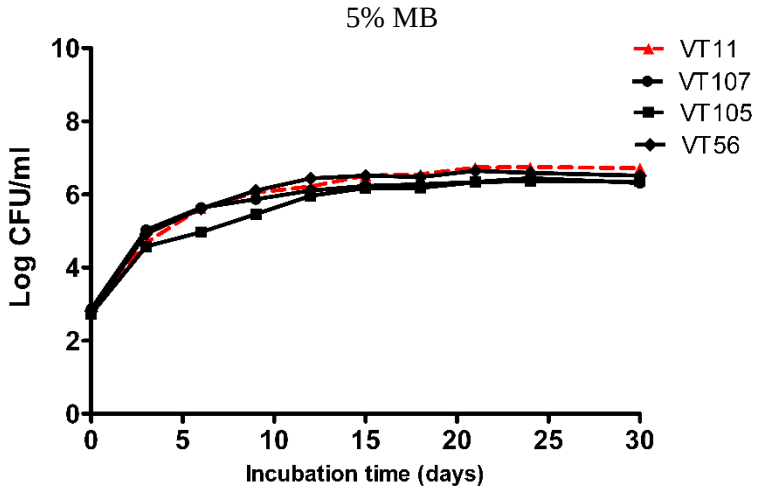
35°C	10°C
TSBYE 	TSBYE 
30% MB 	30% MB 

35°C	10°C
 10% MB VT11 VT107 VT105 VT56 Log CFU/ml 0 2 4 6 8 10 0 30 60 90 120 150 180 210 240 Incubation time (hours)	 10% MB VT11 VT107 VT105 VT56 Log CFU/ml 0 2 4 6 8 10 0 5 10 15 20 25 30 Incubation time (days)
 5% MB VT11 VT107 VT105 VT56 Log CFU/ml 0 2 4 6 8 10 0 30 60 90 120 150 180 210 240 Incubation time (hours)	 5% MB VT11 VT107 VT105 VT56 Log CFU/ml 0 2 4 6 8 10 0 5 10 15 20 25 30 Incubation time (days)

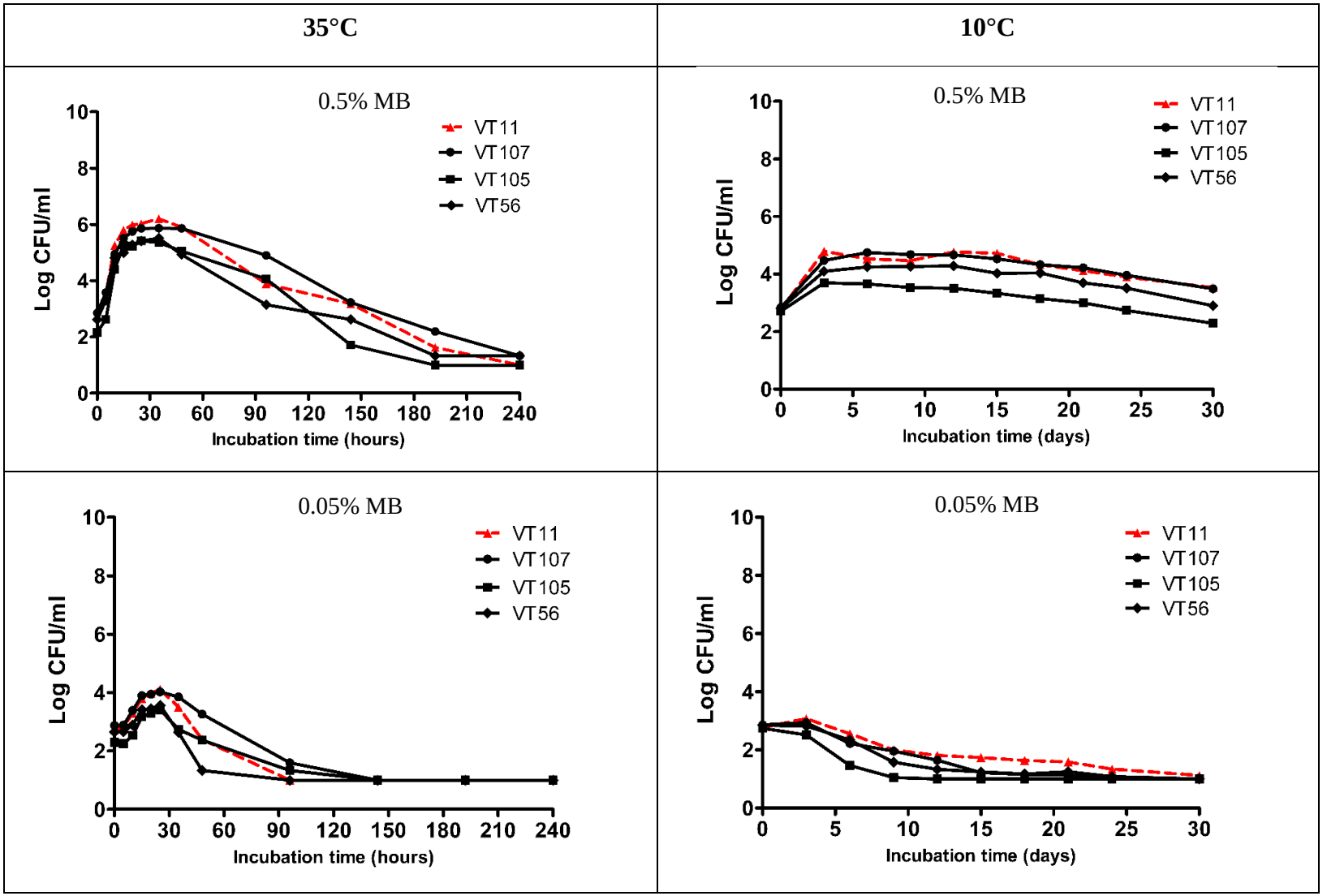


FIGURE 12: Peak cell densities of *L. monocytogenes* clones in TSBYE and MB dilution levels (30%, 10%, 5%, 0.5%, and 0.05%) at 35°C and 10°C.

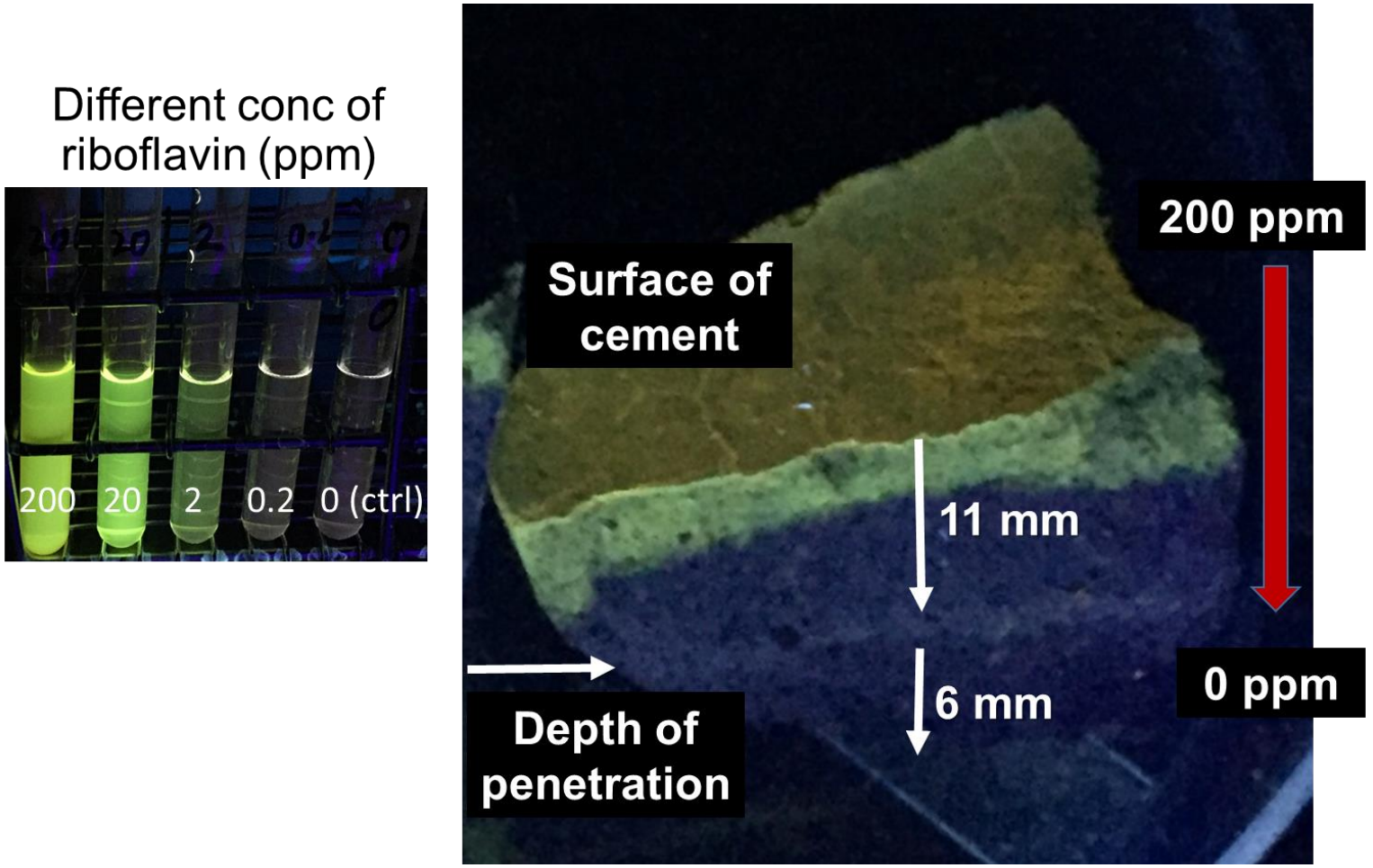


FIGURE 13: Penetration of riboflavin into the model cement slab after 2 h at room temperature under UV light at 365 nm.

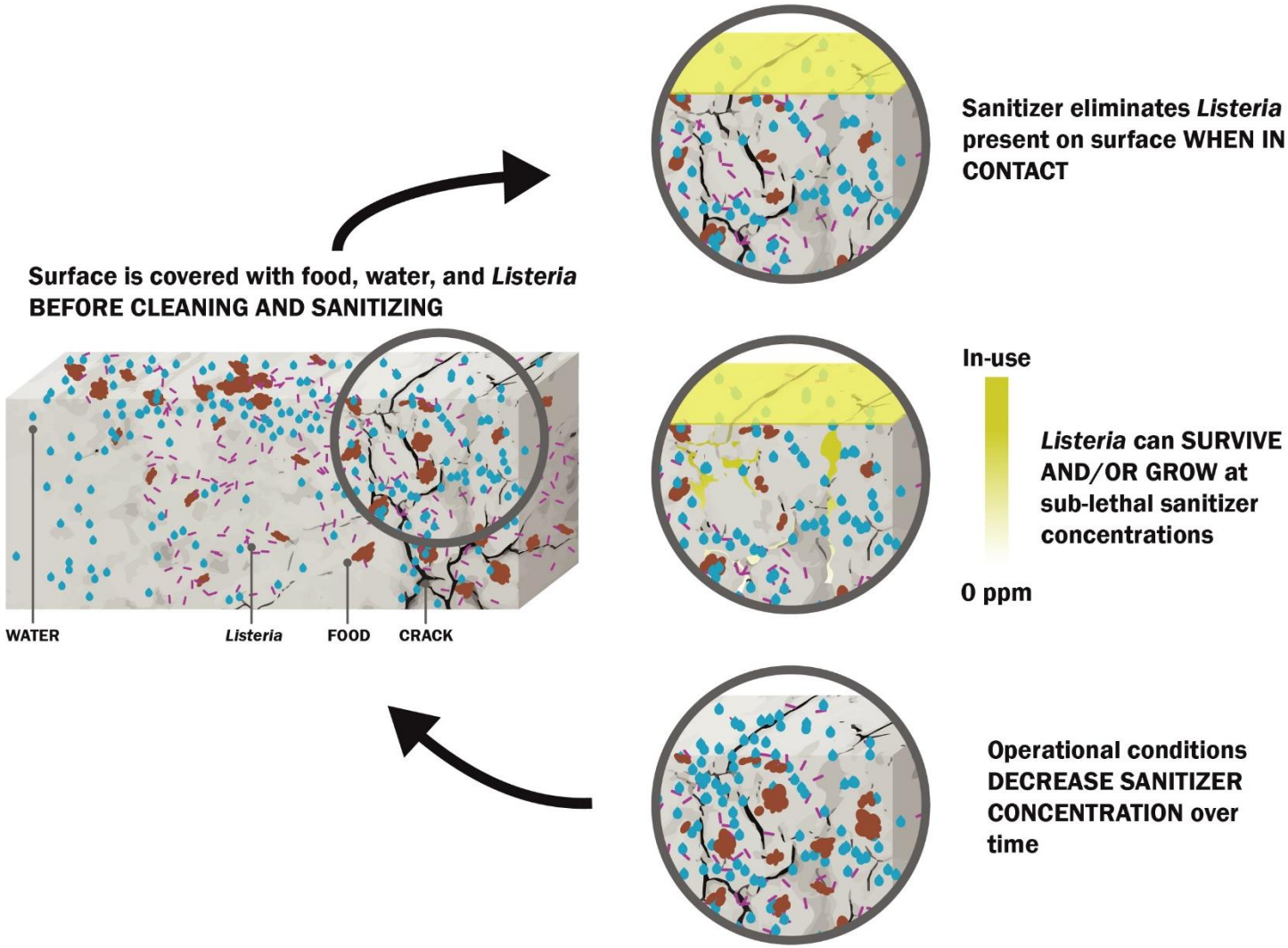


FIGURE 14: A model to explain the contamination of the predominant and persistent *L. monocytogenes* clone (VT11) from a porous concrete or cement floor in the commercial mushroom processing facility

TABLE 8. Generation times of *L. monocytogenes* clones at different mushroom broth dilution levels and TSBYE at 35°C and 10°C.

		Generation time (min)[1]					
			Mushroom broths				
Temperature	**Virulence type**	**TSBYE**	**30%**	**10%**	**5%**	**0.5%**	**0.05%**
35°C	VT11	41.80^{aB}	53.11^{aB}	43.54^{abB}	49.51^{aB}	52.27^{bB}	191.69^{aA}
	VT107	39.39^{aC}	47.50^{aC}	44.84^{abC}	46.46^{aC}	66.95^{aB}	180.57^{aA}
	VT105	40.82^{aB}	51.05^{aB}	40.01^{bB}	40.87^{aB}	53.33^{bB}	161.82^{aA}
	VT56	38.45^{aB}	43.46^{aB}	46.39^{aB}	47.56^{aB}	60.80^{abB}	207.06^{aA}
		Generation time (h)					
10°C	VT11	10.03^{aA}	10.12^{aA}	10.00^{aA}	12.75^{aA}	10.74^{aA}	28.42[2]
	VT107	9.27^{aA}	$11.\ 69^{aA}$	12.50^{aA}	12.33^{aA}	18.92^{aA}	NG
	VT105	10.19^{aA}	15.46^{aA}	12.81^{aA}	14.90^{aA}	25.75^{aA}	NG
	VT56	10.07^{aB}	11.48^{aB}	10.95^{aB}	10.35^{aB}	18.40^{aA}	NG

[1]Each data point represents mean and standard deviation from 3 replicates. Different lower case letters within a column and upper case letters within a row are significantly different ($P \leq 0.05$).

[2]Growth was only observed in one of the three replicates. NG- no growth was observed.

TABLE 9. Peak cell densities of *L. monocytogenes* clones grown in TSBYE and different mushroom broth dilution levels at 35°C and 10°C.

		Log CFU/ml[1]					
			Mushroom broths				
Temperature	**Virulence type**	**TSBYE**	**30%**	**10%**	**5%**	**0.5%**	**0.05%**
35°C	VT11	8.43 ± 0.21^{aA}	5.75 ± 0.58^{aD}	$6.61 \pm 0.02^{cC*}$	$7.22 \pm 0.39^{aB*}$	4.27 ± 0.34^{aE}	2.30 ± 0.09^{abF}
	VT107	8.32 ± 0.15^{aA}	5.29 ± 0.23^{aD}	$6.47 \pm 0.14^{cC*}$	$7.14 \pm 0.30^{aB*}$	4.33 ± 0.56^{aE}	$2.46 \pm 0.20^{aF*}$
	VT105	8.42 ± 0.17^{aA}	4.87 ± 0.39^{bD}	$6.77 \pm 0.11^{bC*}$	$7.28 \pm 0.26^{aB*}$	3.60 ± 0.57^{aE}	$2.02 \pm 0.62^{abF*}$
	VT56	8.28 ± 0.04^{aA}	5.40 ± 0.85^{abC}	$6.95 \pm 0.14^{aB*}$	$7.19 \pm 0.22^{aB*}$	3.65 ± 0.25^{aD}	1.92 ± 0.26^{bE}
10°C	VT11	$8.80 \pm 0.15^{bA*}$	5.81 ± 0.63^{aD}	6.11 ± 0.44^{aC}	6.55 ± 0.11^{aB}	4.35 ± 0.42^{aE}	1.87 ± 0.87^{aF}
	VT107	$9.03 \pm 0.01^{aA*}$	5.50 ± 1.21^{aC}	6.13 ± 0.74^{aB}	6.28 ± 0.15^{bB}	4.36 ± 0.45^{aE}	1.59 ± 0.30^{abF}
	VT105	8.45 ± 0.19^{cA}	5.07 ± 1.75^{bD}	5.62 ± 1.31^{bC}	6.23 ± 0.61^{bB}	3.21 ± 1.35^{bC}	1.23 ± 0.10^{cD}
	VT56	$8.71 \pm 0.43^{bcA*}$	$5.94 \pm 0.54^{aC*}$	6.15 ± 0.35^{aC}	6.53 ± 0.03^{aB}	3.93 ± 0.48^{bD}	1.53 ± 0.29^{bE}

[1]Each data point represents mean and standard deviation from 3 replicates. Different lower case letters within a column and upper case letters within a row are significantly different ($P \leq 0.05$). *indicates that log CFU/ml at a given temperature was significantly higher the corresponding log CFU/ml ($P \leq 0.05$).

TABLE 10: Recovery of VT11 cells from stainless steel and concrete coupons after surface sanitizing with 580 ppm QAC for 10 min at room temperature.

		Log CFU/cm^2			
		Stainless steel		**Concrete**	
Surface		1st incubation	2nd incubation	1st incubation	2nd incubation
Exterior	Untreated	5.99 ± 0.13^{a}	6.81 ± 0.03^{b}	5.63 ± 0.08^{a}	4.93 ± 0.89^{a}
	Treated	ND	ND	ND	ND
Interior*	Untreated	2.26 ± 0.77^{a}	3.06 ± 0.18^{a}	5.06 ± 0.14^{aA}	5.45 ± 1.03^{aA}
	Treated	ND	ND	3.60 ± 0.82^{aB}	2.69 ± 0.09^{aB}

[1]Each data point represents mean and standard deviation from 2 replicates. Different lower case letters within a column and upper case letters within a row are significantly different ($P \leq 0.05$). ND- not detected with a detection limit of 0.6 log CFU/cm^2.
*Interior indicates cells obtained after bead vortexing.

Chapter 6

Summary and questions for future research

6.1 Summary

In the present study, the prevalence of *L. monocytogenes* and other *Listeria* spp. from non-food-contact surfaces were determined in a commercial fresh mushroom processing facility on 3 sampling occasions over a 13- month period. *L. monocytogenes* was found in 18.8% of samples, 4.3% for *L. innocua*, and 2.0% for *L. grayi*. *L. monocytogenes* was most often found on wet floors within the washing and slicing and packaging areas. Multi-virulence-locus sequence typing (MVLST) identified 4 virulence types (VTs): VT11 as the persistent and predominant clone whereas VT107, VT105, and VT56 were classified as transient clones within the facility. A trench drain and wet concrete floor were identified as harborage sites for VT11 in the mushroom processing facility. Significant reduction in *L. monocytogenes* was observed after improvements were made in sanitation practices and facility maintenance between the second and third sampling periods. Nevertheless, VT11 was still isolated on the third sampling occasion during operation. Therefore, VT11 and other clones isolated from the mushroom processing facility were evaluated for their ability to tolerate quaternary ammonium compound (QAC) sanitizer, firmly adhere to surfaces, and grow and maintain a higher peak cell density.

All *L. monocytogenes* clones had the same MIC value of 9.1 ppm; however, the OD_{600} values for VT11 and VT107 were significantly ($P \leq 0.05$) higher than VT105 and VT56 at sub-lethal QAC concentrations. The long-term-survival phase cells of VT11

were significantly ($P \leq 0.05$) more tolerant to in-use QAC concentration of 200 ppm, compared to the three transient clones. Clones were not significantly ($P \leq 0.05$) different in firmly adhered populations on concrete; however, over time, VT11 populations significantly ($P \leq 0.05$) decreased on stainless steel (SS) compared to other clones suggesting an early dispersal from SS surface. Additionally, VT11 grew to a significantly ($P \leq 0.05$) higher peak cell density than other clones at low mushroom broth concentrations suggesting that it is better adapted to a low mushroom nutrient environment. These characteristics of VT11 could provide it with a competitive advantage over transient clones. Based on all results, a model was proposed to explain the persistence of a predominant VT11 clone in the mushroom processing facility. The proposed model may also explain the persistence of certain *L. monocytogenes* subtypes reported by earlier studies conducted in other food processing facilities. This information could be used for developing control strategies to eliminate *L. monocytogenes* from mushroom and other food processing facilities.

6.2 Questions for future research

1. What is the prevalence of *L. monocytogenes* in other commercial mushroom processing facilities? Is VT11 the persistent clone in these facilities?

The present study determined the prevalence and persistence of *L. monocytogenes* from a single commercial mushroom processing facility. However, a comprehensive survey of several mushroom processing facilities will provide information regarding the persistent and transient *L. monocytogenes* clones in other facilities. This information could provide insights on clones that may be shared between facilities and help in developing intervention strategies to eliminate or control *L. monocytogenes* contamination from mushroom processing facilities (See appendix B for preliminary data on isolates obtained from 2 other mushroom processing facilities in the US and 3 mushroom production and processing facilities from non-US sources).

2. Does addition of nutrients such as glucose, amino acids, and/or essential vitamins to the growth medium allow germination and outgrowth of LTS phase *L. monocytogenes* cells? Does this addition of nutrients result in increased sensitivity to sanitizers?

The results from chapter 4 revealed that LTS phase cells were more tolerant to QAC than the 20-h old cells. LTS phase cells have been suggested to be in a non-replicating minimal metabolic activity state. Therefore, if LTS phase cells are allowed to germinate and outgrow to log or lag phase (replicating and increased metabolic activity state) through the addition of glucose, amino acids, and/or essential vitamins, they can be

rapidly killed by the sanitizer. This mechanism could be used for developing intervention measures to reduce *L. monocytogenes* contamination. This can be tested by adding different concentrations of glucose, amino acids, and/or essential vitamins, and expose the LTS phase cells to QAC sanitizer.

3. Does VT11 possess some genetic characteristics resulting in increased QAC tolerance?

The results from chapter 4 demonstrated that 20-h and 7 day old VT11 cells were significantly more tolerant to QAC sanitizer. Previous studies have reported that subtypes containing efflux pumps, plasmids with heavy metal resistance, *bcrABC* cassette, transposon *Tn6188*, *mdrL*, and some *qac* genes were more tolerant to QAC than subtypes that lack these genetic characteristics. Therefore, the presence and upregulation of these genetic elements might provide a competitive advantage for VT11 over other clones. This can be tested by identifying these genetic elements through PCR and quantifying their expression through qPCR.

4. Why are VT11 LTS phase cells more tolerant to QAC than other *L. monocytogenes* clones?

The results from chapter 4 demonstrated that LTS phase cells of VT11 were significantly ($P \leq 0.05$) more tolerant to QAC compared to other clones. A previous study showed that LTS phase cells possess a different transcriptomic profile compared to cells in other phases. Some of the genes that were upregulated are involved in the

transportation of compatible solutes, protection against heat shock, and peptidoglycan synthesis. Variations in expression of these protective genes between the persistent and transient clones might help explain the increased QAC tolerance by VT11. This can be tested by quantifying the expression of the certain genes including *treB, glpF-2, and dnaK* through qPCR.

5. What signaling molecules are causing VT11 to transition to LTS phase sooner than other clones?

The results from chapter 4 suggested that VT11 may transition to LTS phase sooner compared to other clones. In a previous study by Doan et al., (2014) LTS phase cells were reported as persisters. Therefore, it would be interesting to determine whether the mechanisms involved in LTS phase transition and persister cell formation are related or interconnected. The activation of the stress alarmone, ppGpp induces toxin-antitoxin systems resulting in increased toxin production, which cause cells to enter a dormant or minimal metabolically active state. Therefore, the differences in the amount of production of ppGpp and/or toxin between VT11 and other transient clones can be measured using thin layer chromatography and SDS-PAGE which may then provide some explanations for the early transition to LTS phase.

6. Does sealing floors prevent penetration of *L. monocytogenes* within concrete floors?

The results from chapters 3, 4, and 5 demonstrated that QAC surface sanitization did not eliminate *L. monocytogenes* present deep within the concrete. Therefore, sealing

of the concrete with an epoxy or a polyester floor sealant might prevent *L. monocytogenes* from penetrating within the concrete floors, resulting in the elimination of harborage sites. This can be tested by sealing concrete coupons and then evaluating the adherence and penetration abilities of VT11 and other clones.

7. Can multiple hurdle technologies or synergism between antimicrobial compounds destroy firmly adhered *L. monocytogenes* cells on concrete?

The results from chapter 4 demonstrated that firmly adhered cells on concrete showed the lowest log reduction compared to SS. It was also clear that surface sanitizing using QAC was not sufficient to destroy *L. monocytogenes* cells present deep within the concrete. Therefore, in addition to sealing concrete, different antimicrobial compounds may also be used to reduce *L. monocytogenes* contamination. Ethylenediamine tetraacetic acid (EDTA) and peracetic acid were shown to be effective in eliminating biofilms of *L. monocytogenes*. Therefore, a combination of these antimicrobial compounds with different modes of action may work in synergy and allow increased contact between *L. monocytogenes* cells and sanitizers resulting in increased reduction of firmly adhered *L. monocytogenes* cells on concrete.

8. Can VT11 outcompete other clones when incubated together in low mushroom broth dilution levels?

The results from chapter 5 suggest that VT11 can grow to a higher peak cell density in a mushroom nutrient-limited environment. Within concrete floors, it is more

likely the mushroom nutrients could be diluted resulting in a low nutrient environment. Therefore, it will be interesting to determine whether VT11 can outcompete the other transient clones in a low nutrient environment that may result in predominance and persistence. This can be tested by co-incubating VT11 with other clones in 0.5% and 0.05% MB dilutions and performing propidium monoazide (PMAxx) qPCR with primers specific for each clone or serotype. The main advantage of PMAxx - qPCR over regular qPCR is that PMAxx can bind to the DNA of dead cells and prevent DNA from being amplified during qPCR.

9. Are persistent clones of *L. monocytogenes* adapted to the nutrients in the food matrix from which they were originally isolated?

Previous studies have reported that certain subtypes predominate and persist in food processing facilities. The results from this study demonstrated that the persistent and predominant clone may be adapted to the mushroom nutrients (in a nutrient-limited environment) compared to transient clones. Therefore, it might be interesting to understand whether persistent subtypes are in general adapted to the nutrients from which they were originally isolated from. This can be tested by growing persistent subtypes from food facilities in different food matrices and determine the PCDs of *L. monocytogenes* subtypes in each of the food matrices.

Appendix A

Effect of addition of glucose and elimination of background microorganisms on peak cell density of *L. monocytogenes*

Objective: To determine the effect of addition of glucose and elimination of background microorganisms on growth and peak cell density of *L. monocytogenes*.

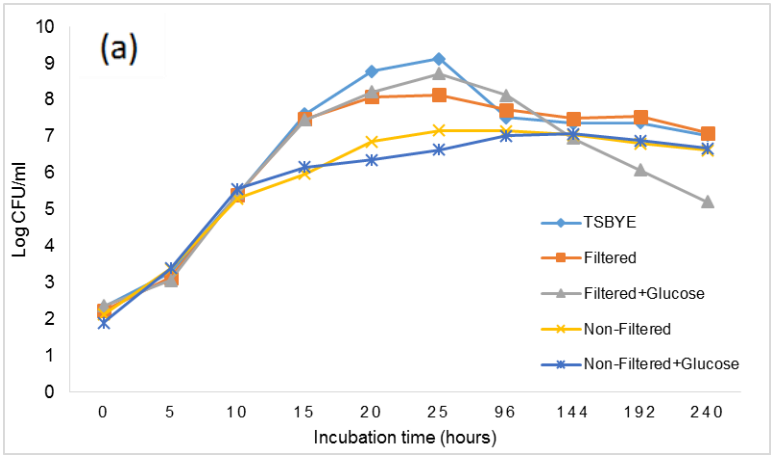
Materials and methods: *L. monocytogenes* clones (VT11, VT107, and VT105) isolated from the wet processing areas of the mushroom processing facility were grown in 10 ml TSBYE for 20 h at 35°C. The cultures were centrifuged at 12,000 rpm for 3 min at 20°C, and the pellets were washed and re-suspended in PBS.

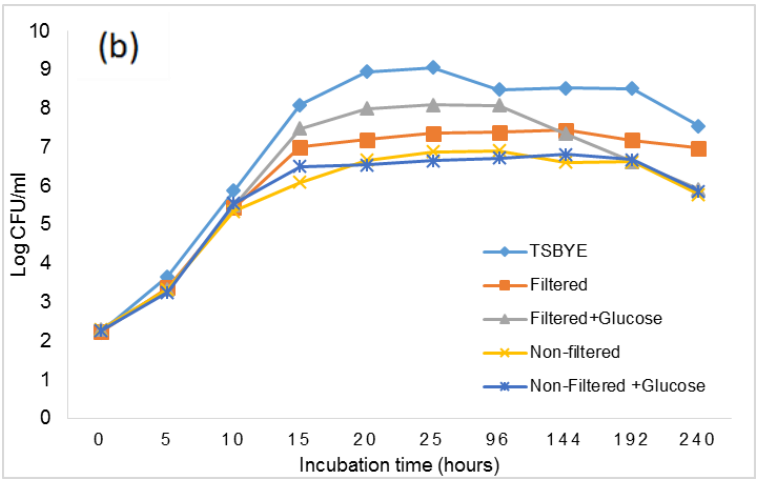
5%MB was prepared by grinding 5 g fresh whole white button mushrooms and 95 g PBS for 1 min. Four different types of treatments were used: fresh MB, filter-sterilized (FS) MB, fresh MB + glucose, filter-sterilized MB + glucose. For filter-sterilized MB, 5% fresh MB was centrifuged at 12,000 rpm for 5 min at 20°C. The supernatant was sterilized using a 0.22 µm filter (VWR International, Radnor, PA, USA). For added glucose treatments, 2.5 ml of 10% glucose (BD Diagnostics Systems) solution was added to 100 g of MB samples to achieve a final concentration of 0.25% which is the same as TSBYE.

One ml of the diluted culture was added to 100 ± 1 g of samples respectively, to achieve a final concentration of ~10^3 CFU/ml. MBs inoculated with individual *L. monocytogenes* cultures were then incubated at 35°C for 10 days. Aliquots were taken at appropriate time intervals and were serially diluted with PBS. The samples were then

plated in duplicate on oxford agar plates supplemented with modified anti-microbic supplement (MOX) (BD Diagnostic Systems). The plates were incubated at 35°C for 48 h before enumeration. Samples inoculated in TSBYE were served as controls. The experiment was performed once.

Results and discussion: FIGURE 15 shows that all *L. monocytogenes* clones grew to a higher PCDs in FS-MB and FS-MB + glucose samples suggesting that the elimination of background organisms and addition of glucose increased PCDs of *L. monocytogenes*. In some instances, for VT11 and VT105, PCDs of FS-MB and FS-MB + glucose samples were the same as TSBYE. However, the populations in FS-MB + glucose decreased more rapidly for all clones compared to other treatments (FIGURE 15**)**. This could be due to the production of acid from glucose utilization resulting in acidic pH of the growth medium and/or preventing *L. monocytogenes* cells to enter the LTS phase, thus decreasing the tolerance to starvation stress and resulting in cell death. On the other hand, for all clones, the addition of glucose to fresh MB did not increase the PCD (FIGURE 15). This might be due to the presence of background organisms that compete with *L. monocytogenes* for nutrients. In conclusion, the added glucose and elimination of competition can provide more available nutrients for growth and higher PCD for all *L. monocytogenes* clones.





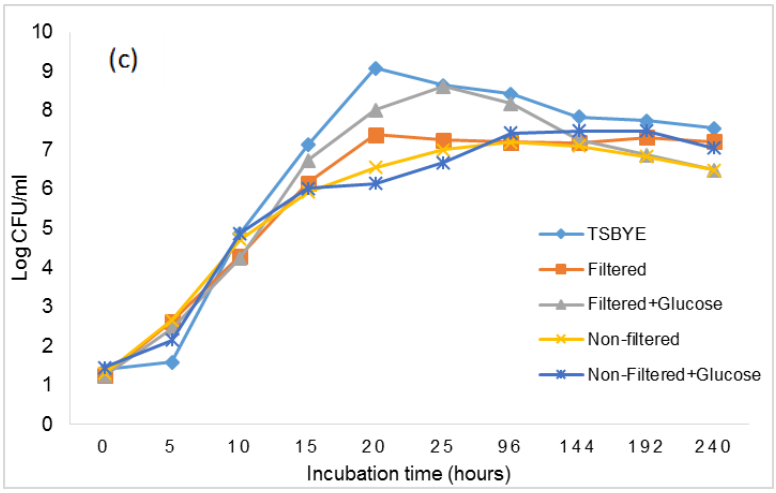


FIGURE 15. Effect of addition of glucose and elimination of background microorganisms on growth and peak cell density of *L. monocytogenes* clones (a) VT11, (b) VT107, and (c) VT105 at 35°C for 10 days.

Appendix B

Diversity of *L. monocytogenes* isolates from other mushroom processing facilities

Objective: To determine the diversity of *L. monocytogenes* isolates obtained from other mushroom processing facilities and to identify any unique universal clones within mushroom processing facilities.

Materials and methods: All *L. monocytogenes* isolates were grown in tryptic soy broth supplemented with 0.6% yeast extract (TSBYE) for 20 h. DNA was extracted using Ultraclean Microbial DNA Isolation kits (Mo Bio Laboratories Inc., Carlsbad, CA). *L. monocytogenes* isolates were serogrouped using the multiplex PCR procedure of Doumith et al., (2004a) and multi-virulence-locus sequence typing was performed as described by Murugesan et al., (2015); Zhang et al., (2004). Amplicons from MVLST were cleaned using ExoSAP-IT (Affymetrix, Santa Clara, CA) and were sent to The Genomics Core facility (The Pennsylvania State University, University Park Campus) for forward and reverse sequencing. The gene sequences were aligned, concatenated, and analyzed using molecular evolutionary genetic analysis software (MEGA 6.0, Tempe, AZ). An unrooted neighbor-joining tree with bootstrap values of 1,000 was constructed to allow ease of subtype comparison between isolates.

Results and discussion: TABLE 11 shows the serogroups and virulence types of *L. monocytogenes* isolated in mushrooms, mushroom processing, and production facilities from the US and non-US sources. VT11 was also isolated from another

mushroom processing facility that is widely separated in space and time in the US in 2015. VT11 was isolated from the facility that was surveyed earlier in this study during 2012 – 2013, indicating that VT11 is still persisting in this mushroom processing facility. It is surprising that four of the *L. monocytogenes* isolates obtained from whole or sliced mushrooms in 2009 from the *Listeria* collection (Dr. LaBorde lab) were also VT11. Nevertheless, VT11 was not isolated from any of the non-US sources (TABLE 11; FIGURE 16). This difference in clonal distribution or selection between the US and non-US sources might be due to the different type of processing methods employed. There is very limited information regarding *L. monocytogenes* isolates analyzed in this experiment and therefore, relationships between these clones cannot be determined.

TABLE 11: Serogroups and virulence types of *L. monocytogenes* isolates detected in mushrooms, mushroom processing, and production facilities from the US and non-US sources**.**

Isolate ID	**Serogroup**	**Virulence type**	**Source**
US origin			
DOD-1	1/2c or 3c	VT11	Whole mushrooms, 2009
DOD-2	1/2c or 3c	VT11	Sliced mushrooms, 2009
DOD-3	1/2c or 3c	VT11	Sliced mushrooms, 2009
DOD-4	1/2c or 3c	VT11	Sliced mushrooms, 2009
DOD-5	1/2a or 3a	VT117	Whole mushrooms, 2009
DOD-6	1/2a or 3a	VT122	Mushrooms, 2009
M3-001	1/2c or 3c	VT11	Environment, Facility 1, 2015 (environment of the facility surveyed earlier in 2012 -2013)
M3-002	1/2a or 3a	VT56	Environment, Facility 2, 2015
M3-003	1/2c or 3c	VT11	Environment, Facility 3, 2015
Non-US origin			
1	1/2b or 3b	VT119	Environment, Facility 1, July 29th 2014
4	1/2a or 3a	VT118	Environment, Facility 5, July 31st 2014
7	1/2a or 3a	VT116	Environment, Facility 5, July 24th 2014
8	1/2a or 3a	VT4	Mushrooms, Facility 1, Oct 6th2014
9	1/2a or 3a	VT61	Mushrooms, Facility 1, Oct 6th2014
10	1/2a or 3a	VT74	Mushrooms, Facility 2, July 30th 2014
12	1/2a or 3a	VT116	Environment, Facility 5, July 31st 2014
13	1/2b or 3b	VT120	Environment, Facility 5, July 24th 2014
14	1/2a or 3a	VT116	Environment, Facility 5, July 24th 2014
15	1/2a or 3a	VT116	Environment, Facility 5, July 24th 2014
21	1/2a or 3a	VT116	Environment, Facility 5, July 24th 2014
B	1/2a or 3a	VT116	Environment, Facility 5, July 24th 2014

*DOD- Department of Defense.

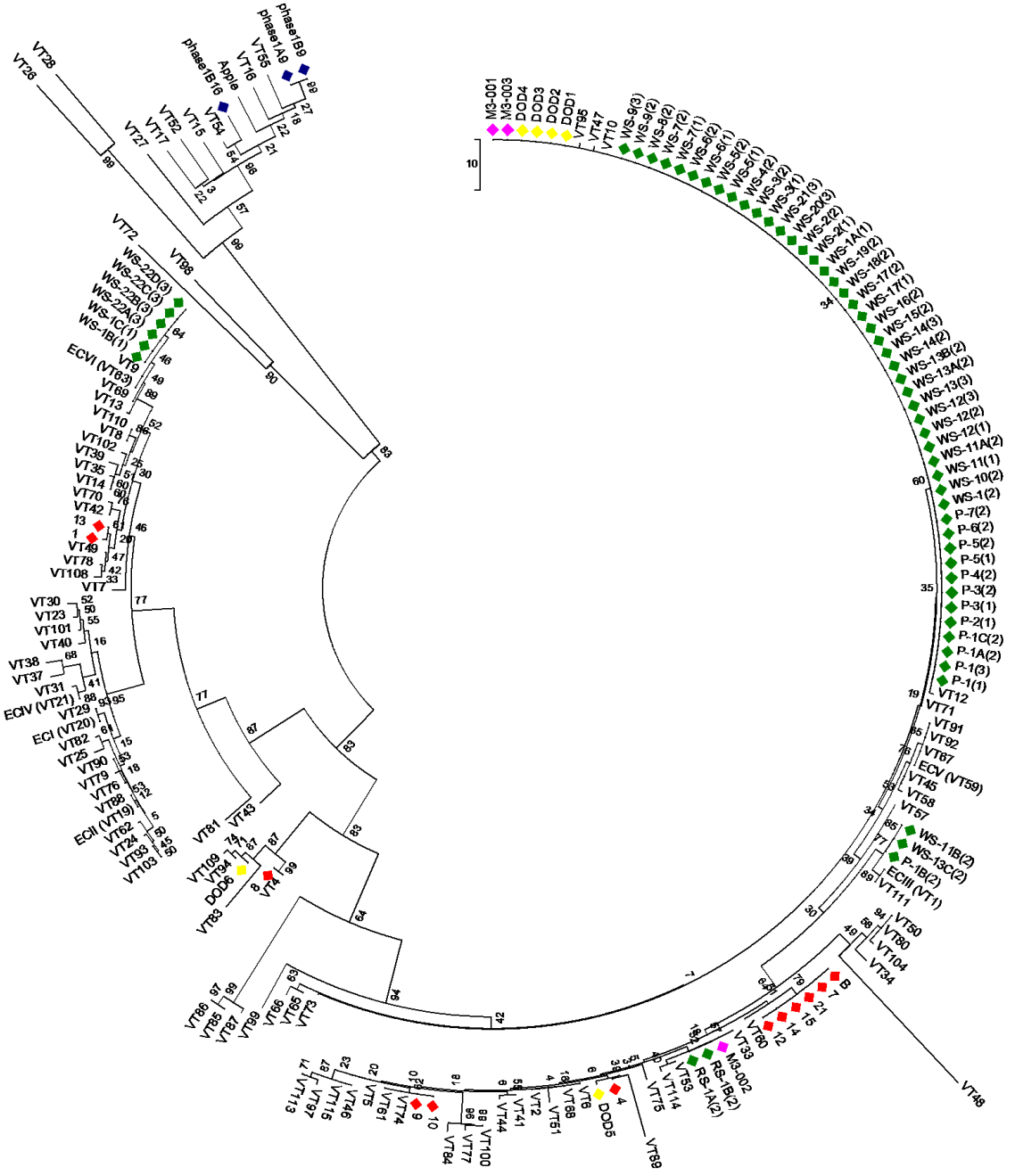


FIGURE 16: Phylogenetic relationship of *L. monocytogenes* isolates from mushrooms, mushroom production, and processing environment isolates.

The US source isolates are listed below: green colored isolates are isolated by Murugesan et al. (2015) from the mushroom processing facility, blue colored isolates are obtained by Viswanath et al. (2013) from a mushroom production environment, pink colored isolates are obtained by routine *Listeria* environmental program employed by 3 mushroom processing facilities, yellow colored isolates are from the collection of Dr. LaBorde's

L.monocytogenes isolates from mushrooms. The non-US source *L. monocytogenes* isolates are in red color which include isolates obtained from mushroom, mushroom production and processing environments.

Appendix C

Developing *L. monocytogenes* serotype specific primers

Objective: To develop specific primer sets for differentiation of 1/2a and 1/2c serotypes using qPCR during co-incubation study.

Materials and methods: DNA of *L. monocytogenes* clones, VT11 (serotype 1/2c) and VT107 (serotype 1/2a) were extracted using Ultraclean Microbial DNA Isolation kits (Mo Bio Laboratories). Current primers used for identification of these serotypes cannot differentiate between these 2 serotypes. Therefore, a new set of primers were developed to differentiate these two serotypes for co-incubation studies using qPCR. Results from Doumith et al. (2004) were used as a reference in the selection of serotype specific genes. The reference genomes of *L. monocytogenes* used for primer design were EGDe for 1/2a serotype and SLCC2372 for 1/2c serotype. The primers were designed using Primer BLAST tool from the National Center for Biotechnology Information. To determine the optimum conditions for amplification of serotype specific primers, a gradient PCR was performed with temperatures ranging between 58 to 50°C. PCR reaction volume consisted of 12.5 µl master mix (Qiagen, Valencia, CA, USA), 0.4 µM each primer, 1.0 µl template DNA and the volume made to 25 µl using nuclease-free water. After amplification, the PCR products were separated using 2% agarose gel, stained in ethidium bromide for 30 min and visualized using a UV Trans illuminator (UVP-LLC, Upland, CA, USA).

Results: Of all the primer sequences tested, the primer sets that were specific for 1/2a and 1/2c serotypes of *L. monocytogenes* are shown in TABLE 12. Of the several PCR conditions tested, the optimum reaction conditions for amplification of the serotype-specific primers are shown in TABLE 13.

TABLE 12: Nucleotide sequences of primer sets developed for differentiation of 1/2a and 1/2c serotypes for qPCR co-incubation assays.

Gene target	Primer sequence (5'- 3')	Product size (bp)	Serotype specificity	References
lmo2305	Forward: TGAAAACTACAGCCTTCGAGAC Reverse: ACGTAACGCAGGGCTAACA	73	1/2a	This study
lmo1118	Forward: GCTCAGCAATTGCGTAACAT Reverse: TGGGCGGAAATTACAATTCGTG	87	1/2c	This study

TABLE 13: Optimized PCR conditions for amplification of serotype specific primers for qPCR.

PCR steps	**Temperature**	**Time**	**Cycles**
Initial denaturation	94°C	3 min	1
Denaturation	94°C	20 s	35
Annealing	57°C	30 s	
Elongation	72°C	25 s	
Final extension	72°C	10 min	1

VITA

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EDUCATION

Penn State, Doctor of Philosophy, Food Science, University Park, PA	2012 - 2016
Chapman University, Master of Science, Food Science, Orange, CA	2008 - 2011
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WORK EXPERIENCE

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HONORS AND SCHOLARSHIPS

- **Honorary mention**, IFTSA & Mars PD competition, 2016
- **2nd place,** National Dairy Council PD competition, 2015
- **3rd place,** IFTSA and Mars PD competition, 2015
- Penn State Food Industry Group **graduate student leadership award,** 2015
- Penn State College Bowl team advisor, 2015, 2014
- Penn State College of Ag Sciences, Janet G. & Frank J. Dudek scholarship, 2015 - 2013
- Team captain, **1st place** IFTSA National & North-Atlantic college bowl championship, 2014
- Team captain, **2nd place** IFTSA North-Atlantic college bowl competition, 2013, 2012
- IFT Extension & Outreach Division scholarship for work on global food safety & supply, 2013
- **5th place** in College of Ag Sciences- Gamma Sigma Delta research poster presentation, 2013
- ASM student travel scholarship, 2013
- Team captain, **2nd place** IFTSA national college bowl championship, 2011
- Team captain, **1st place** IFTSA Mountain-West college bowl competition, 2011, 2010
- Outstanding dedication & involvement award in Mountain-West IFTSA CB, 2011
- **Outstanding graduate student award** from food science, Chapman University, 2010
- Southern California Institute of Food Technologists (SCIFTS) scholarship in 2010