

Incidence of *Listeria monocytogenes* and *Listeria* spp. in a Small-Scale Mushroom Production Facility

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ABSTRACT

Listeria monocytogenes is a foodborne pathogen of significant concern to the agricultural and food processing industry because of its ability to grow and persist in cool and moist environments and its association with listeriosis, a disease with a very high mortality rate. Although there have been no listeriosis outbreaks attributed to fresh mushrooms in the United States, retail surveys and recalls are evidence that *L. monocytogenes* contamination of mushrooms (*Agaricus bisporus*) can occur. The objective of this study was to determine the prevalence of *Listeria* spp., including *L. monocytogenes*, in a small-scale mushroom production facility on the campus of the Pennsylvania State University in the United States. Of 184 samples taken from five production zones within the facility, 29 (15.8%) samples were positive for *Listeria* spp. Among the *Listeria* spp. isolates, *L. innocua* was most prevalent (10.3%) followed by *L. welshimeri* (3.3%), *L. monocytogenes* (1.6%), and *L. grayi* (0.5%). *L. monocytogenes* was recovered only from the phase I raw material composting area. Isolates of *L. monocytogenes* were confirmed and serotyped by multiplex PCR. The epidemiological relatedness of the three *L. monocytogenes* isolates to those serotypes or lineages frequently encountered in listeriosis infections was determined by multi-virulence-locus sequence typing using six virulence genes, namely, *prfA*, *inlB*, *inlC*, *dal*, *clpP*, and *lisR*. The phylogenetic positions of the three isolates in the dendrogram prepared with data from other isolates of *L. monocytogenes* showed that all isolates were grouped with serotype 4a, lineage IIIA. To date, this serotype has rarely been reported in foodborne disease outbreaks.

Contamination of food products with pathogenic *Listeria monocytogenes* is a major concern to the food industry, consumers, and regulatory agencies (52, 54). This organism is especially problematic because of its high lethal rate in humans and its ability to grow at low temperatures and survive within a wide range of pH and osmotic conditions (13, 37, 48). *Listeria* spp. are capable of existing as saprophytes in soil and decaying vegetation and are ubiquitous in agricultural environments including water, sewage, silage, and animal feces (57). Taxonomically, the genus is composed of six species that can be found in agricultural and food processing environments: *L. monocytogenes*, *L. ivanovii*, *L. seeligeri*, *L. innocua*, *L. welshimeri*, and *L. grayi* (15). *L. monocytogenes* is a facultative intracellular pathogen of humans and animals and is readily found in damp, cool indoor environments such as produce packing houses (49). Based on serological techniques that detect interactions between somatic (O) and flagellar (H)

antigens with the corresponding antisera, *L. monocytogenes* consists of 13 serovars: 1/2a, 1/2b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e, and 7 (47). Based on its virulence potential in mammalian hosts, it has been noted that *L. monocytogenes* serovars 4b, 1/2a, 1/2b, and 1/2c are responsible for over 98% of the isolations from clinical cases of human listeriosis (31, 61). Through genotyping studies, *L. monocytogenes* is grouped under three genetic lineages. Lineage I (LI) contains serovars 1/2b, 3b, 4b, 4d, and 4e, lineage II (LII) contains serovars 1/2a, 1/2c, 3a, and 3c, and lineage III (LIII) contains serotypes 4a, 4b, and 4c (59). Ward et al. (58) have assigned the isolates belonging to the LIIIA subgroup into a separate lineage, LIV, based on multilocus genotyping studies. The continuous presence of water and food debris in and on drains, floors, walls, and processing equipment substantially increases the chance for *L. monocytogenes* to survive and grow and eventually contaminate food and food contact surfaces (2, 3, 8, 14, 24, 33, 40).

L. monocytogenes contamination of ready-to-eat (RTE) meats, soft cheeses, and seafood and deli salads has been implicated in many foodborne disease outbreaks (10, 11, 29, 30, 41). Compared with other food items, fruits and vegetables have been implicated in fewer listeriosis outbreaks (30). However, a recent listeriosis outbreak associated with whole cantaloupes was responsible for 146

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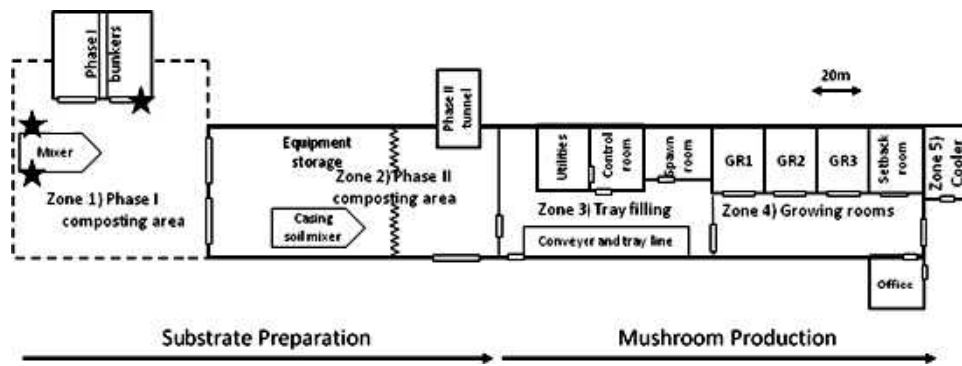


FIGURE 1. Schematic diagram of the Mushroom Test Demonstration Facility (MTDF). Star symbols denote locations of samples confirmed positive for *L. monocytogenes*.

illnesses and 34 deaths (12). Among the possible causes for contamination were the continuous presence of water on floors adjacent to packing equipment, failure to properly clean and sanitize drains and food contact surfaces, and unregulated traffic patterns that may have established routes for *L. monocytogenes* contamination to occur (55). Although there have been no reports of listeriosis directly attributed to consumption of fresh mushrooms, retail surveys indicate that *L. monocytogenes* contamination can occur (32, 46, 56). Several recalls have occurred after detectable levels of *L. monocytogenes* were found on whole or sliced mushrooms. In 2006, 10,000 cases of fresh sliced white mushrooms were recalled after the Ohio Department of Agriculture detected *L. monocytogenes* during random product sampling (53). More recently, the Canadian Food Inspection Agency ordered recalls of Canada-grown mushrooms with detectable levels of *L. monocytogenes* (5–7). These events suggest that *L. monocytogenes* may be entering mushroom production facilities through incoming raw materials and/or surviving and growing in farm and packing house environments.

Successful commercial growing of mushrooms requires steps that include preparation of an organic growth substrate, inoculation of the substrate with *Agaricus bisporus* spawn, crop irrigation, and harvesting (4). Phase I substrate production begins as agricultural raw materials such as straw, horse and poultry manure, corn cobs, brewer's grain, cotton seed, cocoa seed hull, and gypsum are wetted and combined. In phase II, composting conditions have been optimized so that the heat generated by thermophilic microorganisms breaks down complex substrate ingredients into more bioavailable nutrients while simultaneously destroying mushroom disease-causing microorganism and insect pests.

To help maintain the safety of fresh mushrooms, the mushroom industry has proactively partnered with Pennsylvania State University to develop a set of mushroom production food safety standards known as Mushroom Good Agricultural Practices (MGAP) (44). Employee food safety training programs have been developed (39), and general guidance has been issued for preventing *L. monocytogenes* contamination in mushroom growing and packing environments (35). As government regulators and commercial buyers increase their scrutiny of fresh produce

operations, knowledge of the incidence of *L. monocytogenes* in mushroom production environments can be useful for developing intervention strategies that minimize product contamination through targeted sanitation control procedures. In order to begin understanding the potential for *L. monocytogenes* entry and persistence in commercial mushroom facilities, our objective was to survey a small-scale mushroom production facility for the presence of *Listeria* spp., including *L. monocytogenes*.

MATERIALS AND METHODS

Mushroom growing production. This study was conducted at the Mushroom Test Demonstration Facility (MTDF), a small-scale mushroom production facility on the campus of the Pennsylvania State University. The facility is used for mushroom research and educational programming and is also a supplier of campus food service outlets. A diagram of the facility is shown in Figure 1. For the purpose of this study, the MTDF was divided into five zones: zone 1, phase I substrate preparation and composting area; zone 2, phase II composting and equipment storage area; zone 3, tray filling area; zone 4, growing rooms and hallway; and zone 5, cold storage room. The entire area within zone 1 is covered by a roof, although only three sides are walled. Zones 1 and 2 are separated by a retractable heavy plastic curtain. Zones 2, 3, and 4 are separated by permanent indoor walls and are maintained at approximately 10 to 15°C when outside temperatures are below this range. Zone 5 is a walk-in cooler ($\leq 4^{\circ}\text{C}$) adjacent to the MTDF and is accessible only from outside the building.

The equipment and procedures used for growing *A. bisporus* mushrooms are similar to those typically used on commercial farms. In zone I, phase I substrate materials (10.9×10^3 kg) consisting of switchgrass straw, wheat straw-bedded horse manure, kiln-dried poultry manure, distiller's grain, gypsum, and water are mechanically mixed and then transferred using a front-end loader to an adjacent floor-aerated bunker for a 6-day composting treatment. Internal pile temperatures, which are continuously monitored using thermocouples, are allowed to reach up to 80°C. The completed phase I substrate is transferred to an enclosed bulk tunnel facility ($1,700 \text{ m}^3$) in zone 2 for a 6-day phase II composting process. Substrate temperatures, continuously monitored with thermocouples, are adjusted by controlling air flow through the pile. An initial pasteurization step is achieved by allowing internal substrate and surrounding air temperatures to reach 60°C for 2 h, after which the substrate is cooled to 48°C and held at that temperature for the remainder of the process.

After phase II is completed, the substrate is transferred to an automatic conveyor line in zone 3, where it is placed into wooden

TABLE 1. *Mushroom Test Demonstration Facility sampling sites*

MTDF zone	Date of sampling (mo/day/yr)	No. of samples	Sampling sites
1. Phase I composting	3/05/2010	20	Surface of substrate ingredient mixer hopper and conveyor, shovels, pickaxe, hoses, storm water drains, door surfaces, floor surfaces and crevices
	4/01/2010	30	
2. Phase II composting	3/09/2010	14	Phase II tunnel (interior and exterior surfaces), hand tools, adjacent floor and wall surfaces and crevices, covered floor drain
	4/10/2010	16	
3. Tray filling line	3/09/2010	18	Filling line, conveyor belt, floor and wall surfaces, covered and open trench drains
	4/05/2010	22	
4. Growing rooms	3/11/2010	30	Wooden trays, harvesting knives and baskets, floor and wall surfaces and crevices, door surfaces, humidifier, covered floor drain, broom and dust pan, step ladder
	4/06/2010	20	
5. Cooler	4/05/2010	14	Floor, walls, door surfaces, and crevices
Total		184	

trays (1.24 m², approximately 100-kg capacity) with simultaneous addition of nutrient supplements and *A. bisporus* spawn. Mycelium is allowed to grow throughout the substrate for 16 days at 24°C (spawn run), after which the trays are moved back to the conveyor line and a 3- to 4-cm surface layer of casing soil is applied. Prior to this step, the casing soil has been prepared by mixing horticultural peat with crushed limestone, *A. bisporus* spawn, and water. The trays are then moved into temperature-controlled growing rooms (zone 4) where air temperatures are held at 17 to 18°C. After approximately 17 days, mushrooms are harvested by hand (1,500-kg yield per growing room) and immediately transported to campus food service outlets or stored overnight in a refrigerated walk-in storage unit (zone 5). After each crop cycle, the growing rooms are sealed and steamed for 24 h to reduce populations of fungal and bacterial mushroom pathogens and insect pests. Floors and equipment surfaces in zones 2 to 5 are scrubbed with a detergent and rinsed with potable water, followed by application of a sodium hypochlorite (400 ppm) sanitizing solution.

Sample collection. Sampling sites were chosen based on knowledge of environmental conditions most likely to support the survival and growth of *Listeria* spp. as well as points where cross-contamination could occur. The sample sites within the five zones are described in Table 1. Sterile sponges (3M sponge stick, 3M Microbiology, St. Paul, MN) were prepared for sampling at each site by aseptically soaking them in 30 ml of sterile modified University of Vermont broth (BD Diagnostic Systems, Franklin Lakes, NJ) in sterile sample bags. Excess buffer was aseptically squeezed from the hydrated sponge prior to surface sampling. Sterile gloves were used to obtain surface samples within an area of 30.5 by 30.5 cm. Narrow or inaccessible spaces such as floor crevices and drains were sampled along a 30.5-cm length. A total of 184 samples were collected in March and April 2010. The average hourly outside temperature during each sampling period was 4.9 ± 8.5°C and 12.9 ± 11.4°C. All samples were transported on ice to the Department of Food Science for microbial analysis within 30 min of sampling.

Detection of *Listeria* spp. After transportation to the laboratory, an additional 70 ml of sterile modified University of Vermont broth was added to each of the sampling bags and thoroughly mixed. The sample bags were then incubated at 35°C for 24 h. The incubated samples were mixed again and streaked onto modified Oxford agar (BD Diagnostic Systems) plates, which were incubated at 35°C for 24 to 48 h (45). Presumptive isolates of *Listeria* spp. were streaked onto Trypticase soy agar with 0.6%

yeast extract (TSAYE; BD Diagnostic Systems) and then incubated at 35°C for 24 h. The isolates were stored at 4°C as TSAYE slants for Gram staining and microscopic examination. A suspension of each of the isolates was prepared in 0.85% saline and examined for tumbling motility by the hanging drop method. Oxoid Microbact *Listeria* 12 L identification kits (Oxoid Ltd., Basingstoke, Hampshire, UK) were used for identifying individual *Listeria* spp. Isolates were tested for beta-hemolysis and for utilization of 11 carbohydrates (esculin, mannitol, xylose, arabinol, ribose, rhamnose, trehalose, tagatose, glucose-1-phosphate, methyl-D-glucose, and methyl-D-mannose). After incubation, the results were visually determined and interpreted using the Microbact Computer Aided Identification Package. Phosphatidylcholine phospholipase activity of isolates was determined by streaking onto Brilliance *Listeria* agar (Oxoid).

Multiplex PCR. MTDF isolates presumptive for *L. monocytogenes* were analyzed by multiplex PCR assay using the Qiagen Multiplex PCR Plus kit (Qiagen, Inc., Valencia, CA). Six *L. monocytogenes* isolates previously identified as 1/2a, 1/2b, 1/2c, 4a, 4b, and 4c (19, 60) were used as positive controls. A negative control consisted of the reaction mixture with no template DNA. A previously confirmed *L. innocua* isolate was also tested to confirm the specificity of the PCR. For preparation of genomic DNA, *L. monocytogenes* isolates were grown in Trypticase soy broth with 0.6% yeast extract (BD Diagnostics Systems) for 18 h at 35°C and isolated using an UltraClean Microbial DNA Isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA). A modified multiplex PCR procedure was used for simultaneous confirmation of *L. monocytogenes* (18) and identification of *L. monocytogenes* serotypes (26). Multiplex PCR amplification and gel electrophoresis of PCR products were subsequently carried out as previously described by Doumith et al. (26).

MVLST. The multi-virulence-locus sequence typing (MVLST) protocol described by Zhang et al. (60) was performed for each of the three confirmed *L. monocytogenes* isolates. Three virulence genes (*prfA*, *inlB*, and *inlC*) and three virulence-associated genes (*dal*, *lisR*, and *clpP*) were used in the assay. *prfA* encodes the positive master regulator of most of the known virulence genes. *inlB* and *inlC* belong to a multigene family of internalins encoding several extracellular proteins required for invasion of mammalian tissue. The genes *dal*, *lisR*, and *clpP* code for alanine racemase, two-component response regulator, and Clp protease proteolytic subunits, respectively. PCR amplification and gel electrophoresis of PCR products were carried out as previously described by Zhang et al. (60). The amplicons were

TABLE 2. Incidence of *Listeria* spp. in the Mushroom Test Demonstration Facility^a

Zone	No. of samples	No. of isolates	No. of isolates belonging to <i>Listeria</i> sp.:					
			Lm	Lin	Lw	Lg	Ls	Liv
1	50	20	3	13	4	0	0	0
2	30	3	0	2	0	1	0	0
3	40	2	0	2	0	0	0	0
4	50	4	0	2	2	0	0	0
5	14	0	0	0	0	0	0	0
Total	184	29	3	19	6	1	0	0
% prevalence		15.7	1.6	10.3	3.3	0.5	0	0

^a Lm, *L. monocytogenes*; Lin, *L. innocua*; Lw, *L. welshimeri*; Lg, *L. grayi*; Ls, *L. seeligeri*; Liv, *L. ivanovii*.

cleaned by adding 0.5 µl of exonuclease I (10 U/µl; USB Corp., Cleveland, OH) and 0.5 µl of shrimp alkaline phosphatase (1 U/µl; USB Corp.) for every 10 µl of PCR product. The mixture was incubated at 37°C for 45 min to degrade the primers and unincorporated deoxynucleoside triphosphates and then at 80°C for 15 min to inactivate the enzymes. DNA sequencing was carried out with an ABI Prism 3100 DNA sequencer (Applied Biosystems, Inc., Foster City, CA) at the Pennsylvania State University Genomics Core Facility. Data evaluation and editing were carried out using DNA Star software (version 8.1, DNASTAR, Inc., Madison, WI). Multiple sequence alignments were performed with molecular evolutionary genetic analysis software (MEGA version 4.0.2, Tempe, AZ) (50). The neighbor-joining tree building algorithm implemented in MEGA was used to construct a dendrogram showing the evolutionary relationship.

RESULTS AND DISCUSSION

Presumptive *Listeria* spp. were identified based upon growth in the presence of the selective agents in modified Oxford agar and by noting the formation of black zones around colonies due to esculin hydrolysis. Colonies were small, round, low, convex, and 0.5 to 1.5 mm in diameter, and upon prolonged incubation, the colonies became larger, often with a sunken center. Presumptive-positive *Listeria* spp. were gram-positive bacilli, showed typical tumbling motility at 25°C, were nonmotile at 37°C, and were catalase positive and oxidase negative.

Because the locations of samples testing positive for *Listeria* spp. were similar in the March and April sampling periods (data not shown), the results within each MTFDF zone were combined (Table 2). Isolates of *Listeria* were recovered from 29 of the 184 sampling sites, with the greatest frequency of occurrence in zone 1. No *Listeria* spp. were recovered from zone 5, the walk-in cooler. Presumptive *L. monocytogenes* was recovered from only three sampling sites in zone 1 (Fig. 1), and these isolates were coded phase 1 A9, phase 1 B9, and phase 1 B16. Two of these isolates were found at two different places on the mechanical substrate ingredient mixer, and one was found at the base of the phase I bunker wooden door. *L. innocua* was the most frequently detected *Listeria* spp. Thirteen isolates were found on floors and mixing equipment in zone 1, two were recovered from zone 2 drains, two from the surface of a tool and on a plastic sheet on the zone 3 tray-filling line, and two were taken from the floor and rungs of a stepladder in a zone 4 growing room. Four isolates of *L. welshimeri*

were found on the floor in zone 1, and two were found on a dustpan and on a hand tool in one of the zone 4 growing rooms. A single isolate of *L. grayi* was recovered from a windowsill adjacent to the phase II tunnel. *L. ivanovii* and *L. seeligeri* were not found in any zones. The presence of *L. grayi* has rarely been reported from environmental samples (36).

Interestingly, nonpathogenic *Listeria* spp. were detected in zones 2 to 4, although no *L. monocytogenes* isolates were found in these areas. Previous surveys for *Listeria* spp. in food production environments have reported a predominance of *L. innocua* over *L. monocytogenes* (1, 22, 51). It has been suggested that greater recovery of *L. innocua* may be due to more-efficient utilization of nutrients during enrichment culturing (23, 42, 43, 62). Alternatively, it has been proposed that *L. innocua* may inhibit *L. monocytogenes* growth by producing bacteriocins or through a strain-dependent quorum-sensing mechanism (9, 21). However, approximately equal proportions of the two species were found in separate fish processing plants by Eklund et al. (27) and Chen et al. (15). Conversely, Hu et al. (34) did not recover any *L. innocua* from 782 environmental and product samples taken from a cold-smoked fish processing facility. Further studies are needed to determine if the reported variation in proportions of *L. monocytogenes* to *L. innocua* is related to differential growth during the enrichment procedure or if nonpathogenic species of *Listeria* might affect growth and survival of *L. monocytogenes* in agricultural and food processing environments.

Each of the six positive-control *L. monocytogenes* serotypes used in the multiplex PCR assay showed appropriate band length as described in Doumith et al. (26). The negative control and the *L. innocua* isolate did not show any bands (Fig. 2). The three presumptive MTFDF *L. monocytogenes* isolates showed a band length of 420 bp, which confirmed the presence of *L. monocytogenes* (18). However, the absence of other band lengths indicated that these isolates belonged to serotype 4a or 4c (26).

MVLST was conducted to further understand the epidemiological relationship of the three *L. monocytogenes* isolates from the mushroom production environment and to determine their relationship to serotypes or lineages frequently encountered in outbreak events. Of the six virulence genes used in the study, amplification was confirmed for five of them. The primers for internalin C

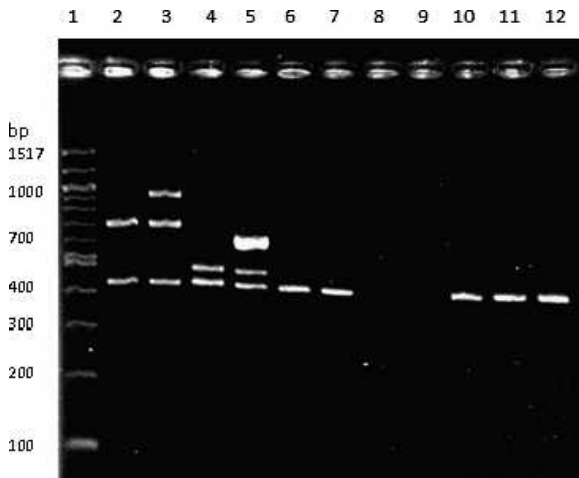


FIGURE 2. Agarose gel electrophoresis results for the multiplex PCR assay of *L. monocytogenes* serotypes. Lane 1, molecular marker, 100 bp; lanes 2 through 7, positive controls (1/2a, 1/2c, 1/2b, 4b, 4c, and 4a); lane 8, negative control; lane 9, *L. innocua*; lanes 10 through 12, MTDf *L. monocytogenes* isolates.

(*inlC*) did not give amplification for any of the three isolates, indicating that it was absent or that the primers failed to bind to template DNA due to nucleotide changes in the primer binding site. The absence of the *inlC* gene in the MTDf isolates is consistent with them being serovar 4a, since this serovar has also been reported to lack this gene (36). Reduced virulence due to lack of this gene has also been observed (28). The phylogenetic positions of the three MTDf *L. monocytogenes* isolates are displayed in the dendrogram in Figure 3. By using National Center for Biotechnology Information (NCBI, Bethesda, MD) data of known *L. monocytogenes* isolates, they were found to be grouped with serotype 4a, LIII. Since these are rhamnose-utilizing strains, they likely belong more specifically to the LIIIA group (38).

Of 2,168 nucleotide sites analyzed, isolate phase 1 B16, taken from the base of a phase I bunker wooden door, showed 0.7% nucleotide diversity with the other two zone 1 *L. monocytogenes* isolates. It is interesting that this particular isolate was obtained from a different location from the other isolates, which were obtained from the substrate ingredient mixing equipment. The nucleotide diversity levels between the isolates from the MTDf facility and the human sporadic case isolates BL0042, BL0046, and BL0045 (19) were 1.0, 0.9, and 0.5%, respectively. The isolate BL0045, which differs from the MTDf isolates by only 0.5%, also belongs to serovar 4a, unlike BL0042, which is serotype 4b. The serotype of isolate BL0046 is not available (19).

Because all three *L. monocytogenes* strains were found in zone 1 and each belongs to the same cluster, it is likely that they are of common origin; possibly horse manure. Kiln-dried poultry manure may not be a likely source of *L. monocytogenes* at the MTDf since the manufacturing process includes a high-temperature drying step. However, raw poultry manure is normally used on commercial farms and should be considered a potential source of *L. monocytogenes* at these locations. A long-term study is

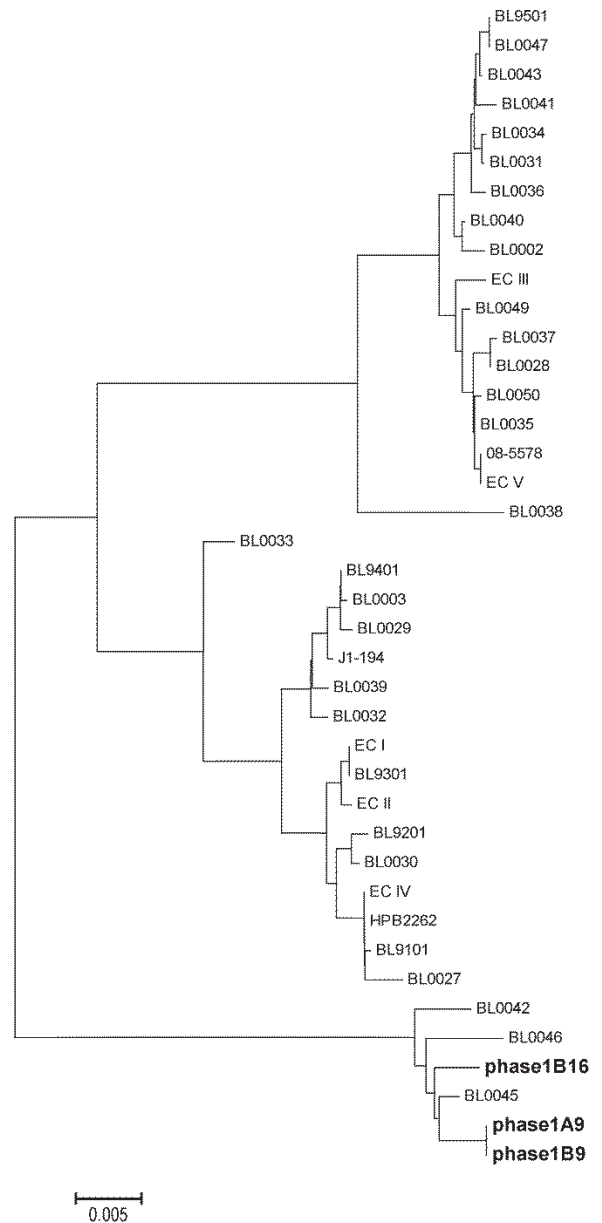


FIGURE 3. Placement of Mushroom Test Demonstration Facility (MTDF) *L. monocytogenes* isolates in the unrooted neighbor-joining tree based on multi-virulence-locus sequence typing (MVLST). Bold isolates are from the MTDf. Nonbold isolates are from author S. J. Knabel's *L. monocytogenes* collection.

necessary to determine if *L. monocytogenes* is consistently limited to raw-material areas in zone 1 or if sporadic contamination throughout the MTDf occurs. Serovar 4a is considered a possible evolutionary intermediate between *L. monocytogenes* serotypes 1/2a and 4b and *L. innocua* (17). Many *L. monocytogenes* LIIIA strains have been found to have identifiable linkage with *L. innocua* by possessing genes common to *L. monocytogenes* (e.g., *Listeria* pathogenicity island I [LPII-1], *inlAB* locus, *bsh*, and *hpt*). They also share gene deletions similar to those of *L. innocua* (e.g., *inlC*, *inlI*, *inlJ*, the internalin cluster between *ascB* and *dapE*, and the arginine deiminase island Imo0036-Imo0041) (16). Perhaps the isolation of *L. innocua* and *L. monocytogenes* IIIA strains from the same environment was more

than a coincidence. Comparative genomic analysis and pangenomic DNA array hybridization studies carried out by Deng et al. (25) have unraveled some of the factors as to why LIII isolates have never been implicated in human disease outbreaks, in spite of the fact that they possess virulence factors. Genes involved in carbohydrate metabolism and transport and for utilization of a wide variety of carbon sources are present in LI and LII strains but not in LIII strains. Similarly, carbohydrate phosphotransferase systems are conserved in LI and LII strains but are lacking in LIII strains. Also, multiple genes involved in combating gastrointestinal tract-related stresses such as gastric acid (*gadD1*, *gadT1*, and arginine deiminase system) and bile salts (*btlB* and *pva*) are missing in LIII strains, thus likely diminishing LIII strain survival in the gastrointestinal tract.

Despite the absence of *L. monocytogenes* in zones 2 to 5, other *Listeria* spp. were found in these areas. Their presence indicates that environmental conditions throughout the MTFD may be conducive to *L. monocytogenes* growth and that sanitation practices should be reviewed. A policy limiting movement of equipment and personnel between MTFD zones was not in place at the time of sampling and therefore should be implemented to prevent cross-contamination of *Listeria* spp. Sanitizer footbaths should also be installed at door entrances. The heavy plastic curtain separating the phase II tunnel and the equipment storage area in zone 2 is probably not adequate to prevent movement of dust particles and water aerosols from zone 1 onto the pasteurized phase II substrate in zone 2 and should be replaced with a more permanent structure. Preparation of casing soil in the equipment storage area is of concern, since it does not receive a microbial reduction treatment and may be ingested by the consumer if not washed off prior to consumption (20). In the light of recovery of *Listeria* spp. from these areas, the cleaning and sanitizing procedures should be evaluated. Floors should be regularly cleaned to remove spilled substrate and casing soil. Sanitation procedures originally developed to suppress fungal species that cause disease in mushrooms should be reevaluated for their effectiveness against *Listeria* spp.

The results of this study have demonstrated that *Listeria* spp., including *L. monocytogenes*, can be found in mushroom growing environments. Each of the *L. monocytogenes* isolates grouped with serotype 4a LIIIA, which has not been reported in foodborne outbreaks in the past. However, finding LIIIA *L. monocytogenes* may indicate that LI and LII organisms might also be able to colonize these environments and contaminate mushrooms. The species of greatest prevalence in the MTFD, particularly in the zone 1 composting area, was *L. innocua*, an evolutionary descendent of *L. monocytogenes* that has lost many virulence genes. Although the MTFD mushroom growing process is similar to that of commercial farms, extrapolation of the results to the entire mushroom growing industry must be done with caution since facilities design, production practices, and adherence to MGAP vary within the industry. A longer-term longitudinal study tracking *L. monocytogenes* at a larger-scale commercial mushroom facility where substrate preparation, growing, packing, and slicing

operations occur would lead to a more complete understanding of the different serotypes and lineages of *L. monocytogenes* present and their reservoirs and routes of transmission. However, this study is of value if evidence for the presence of *Listeria* spp. in the MTFD encourages the industry to refine and improve MGAP sanitation procedures to limit entry, survival, and growth of *Listeria monocytogenes* in mushroom growing environments.

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REFERENCES

1. Aguado, V., A. Vitas, and I. Garcia-Jalon. 2004. Characterization of *Listeria monocytogenes* and *Listeria innocua* from a vegetable processing plant by RAPD and REA. *Int. J. Food Microbiol.* 90:341–347.
2. Arimi, S., E. Ryser, T. Pritchard, and C. Donnelly. 1997. Diversity of *Listeria* ribotypes recovered from dairy cattle, silage, and dairy processing environments. *J. Food Prot.* 60:811–816.
3. Autio, T., S. Hielm, M. Miettinen, A. Sjöberg, K. Aarnisalo, J. Björkroth, T. Mattila-Sandholm, and H. Korkeala. 1999. Sources of *Listeria monocytogenes* contamination in a cold-smoked rainbow trout processing plant detected by pulsed-field gel electrophoresis typing. *Appl. Environ. Microbiol.* 65:150–155.
4. Beyer, D. M. 2003. Basic procedures for *Agaricus* mushroom growing. In College of Agricultural Sciences, extension note. The Pennsylvania State University, University Park. Available at: <http://americanmushroom.org/agaricus.pdf>. Accessed 25 January 2012.
5. Canadian Food Inspection Agency. 2 September 2008. Health hazard: various sliced mushroom products may contain *Listeria monocytogenes*. Available at: <http://epe.lac-bac.gc.ca/100/206/301/cfia-acia/2011-09-21/www.inspection.gc.ca/english/corpaffr/reclarapp/2008/20080801ce.shtml>. Accessed 4 February 2013.
6. Canadian Food Inspection Agency. 10 December 2011. Health hazard alert: certain sliced mushrooms may contain *Listeria monocytogenes*. Available at: <http://www.inspection.gc.ca/english/corpaffr/reclarapp/2011/20111210e.shtml>. Accessed 4 February 2013.
7. Canadian Food Inspection Agency. 13 August 2012. Health hazard alert: certain sliced mushrooms may contain *Listeria monocytogenes*. Available at: <http://www.inspection.gc.ca/english/corpaffr/reclarapp/2012/20120813e.shtml>. Accessed 4 February 2013.
8. Cao, J., M. Clarke, R. Witkowsky, H. Lu, A. Sayedahman, R. E. Levin, and L. A. McLandsborough. 2006. Concentrations and tracking of *Listeria monocytogenes* strains in a seafood-processing environment using a most-probable-number enrichment procedure and randomly amplified polymorphic DNA analysis. *J. Food Prot.* 69:489–494.
9. Carvalheira, A., C. Eusebio, J. Silva, P. Gibbs, and P. Teixeira. 2010. Influence of *Listeria innocua* on the growth of *Listeria monocytogenes*. *Food Control* 21:1492–1496.
10. Centers for Disease Control and Prevention. 2000. Multistate outbreak of listeriosis—United States (2000). *Morb. Mortal. Wkly. Rep.* 49:1129–1152.
11. Centers for Disease Control and Prevention. 2002. Public health dispatch: outbreak of listeriosis—northeastern United States. *Morb. Mortal. Wkly. Rep.* 51:945–964.

12. Centers for Disease Control and Prevention. 2011. Multistate outbreak of listeriosis associated with Jensen Farms cantaloupe—United States. *Morb. Mortal. Wkly. Rep.* 60:1357–1358.
13. Chan, Y., and M. Wiedmann. 2009. Physiology and genetics of *Listeria monocytogenes* survival and growth at cold temperatures. *CRC Crit. Rev. Food Sci. Nutr.* 49:237–253.
14. Chasseignaux, E., P. Gerault, M. Toquin, G. Salvat, P. Colin, and G. Ermel. 2002. Ecology of *Listeria monocytogenes* in the environment of raw poultry meat and raw pork meat processing plants. *FEMS Microbiol. Lett.* 210:271–275.
15. Chen, B., R. Pyla, T. Kim, J. Silva, and Y. Jung. 2010. Incidence and persistence of *Listeria monocytogenes* in the catfish processing environment and fresh filets. *J. Food Prot.* 73:1641–1650.
16. Chen, J., Q. Chen, L. Jiang, C. Cheng, F. Bai, J. Wang, F. Mo, and W. Fang. 2010. Internalin profiling and multilocus sequence typing suggest four *Listeria innocua* subgroups with different evolutionary distances from *Listeria monocytogenes*. *BMC Microbiol.* 10:97.
17. Chen, J., L. Jiang, X. Chen, X. Luo, Y. Chen, Y. Yu, G. Tian, D. Liuz, and W. Fang. 2009. *Listeria monocytogenes* serovar 4a is a possible evolutionary intermediate between *L. monocytogenes* serovars 1/2a and 4b and *L. innocua*. *J. Microbiol. Biotechnol.* 19: 238–249.
18. Chen, Y., and S. Knabel. 2007. Multiplex PCR for simultaneous detection of bacteria of the genus *Listeria*, *Listeria monocytogenes*, and major serotypes and epidemic clones of *L. monocytogenes*. *Appl. Environ. Microbiol.* 73:6299–6304.
19. Chen, Y., W. Zhang, and S. Knabel. 2007. Multi-virulence-locus sequence typing identifies single nucleotide polymorphisms which differentiate epidemic clones and outbreak strains of *Listeria monocytogenes*. *J. Clin. Microbiol.* 45:835–846.
20. Chikthimmah, N., L. LaBorde, and R. Beelman. 2005. Hydrogen peroxide and calcium chloride added to irrigation water as a strategy to reduce bacterial populations and improve quality of fresh mushrooms. *J. Food Sci.* 70:M273–M278.
21. Cornu, M., M. Kalmokoff, and J. P. Flandrois. 2002. Modelling the competitive growth of *Listeria monocytogenes* and *Listeria innocua* in enrichment broths. *Int. J. Food Microbiol.* 73:261–274.
22. Cox, L. J., T. Kleiss, J. L. Cordier, C. Cordellana, P. Konkel, C. Pedrazzini, R. Beumer, and A. Siebenga. 1989. *Listeria* spp. in food processing, non-food and domestic environments. *Food Microbiol.* 6: 49–61.
23. Curiale, M., and C. Lewus. 1994. Detection of *Listeria monocytogenes* in samples containing *Listeria innocua*. *J. Food Prot.* 57:1048–1051.
24. D'Amico, D., and C. Donnelly. 2008. Enhanced detection of *Listeria* spp. in farmstead cheese processing environments through dual primary enrichment, PCR, and molecular subtyping. *J. Food Prot.* 71: 2239–2248.
25. Deng, X., A. Phillippy, Z. Li, S. Salzberg, and W. Zhang. 2010. Probing the pan-genome of *Listeria monocytogenes*: new insights into intraspecific niche expansion and genomic diversification. *BMC Genomics* 11:500.
26. Doumith, M., C. Buchrieser, P. Glaser, C. Jacquet, and P. Martin. 2004. Differentiation of the major *Listeria monocytogenes* serovars by multiplex PCR. *J. Clin. Microbiol.* 42:3819–3822.
27. Eklund, M., F. Poysky, R. Paranjpye, L. Lashbrook, M. Peterson, and G. Pelroy. 1995. Incidence and sources of *Listeria monocytogenes* in cold smoked fishery products and processing plants. *J. Food Prot.* 58: 502–508.
28. Engelbrecht, F., S. Chun, C. Ochs, J. Hess, F. Lottspeich, W. Goebel, and Z. Sokolovic. 1996. A new PrfA-regulated gene of *Listeria monocytogenes* encoding a small, secreted protein which belongs to the family of internalins. *Mol. Microbiol.* 21:823–837.
29. Gilmour, M., M. Graham, G. Van Domselaar, S. Tyler, H. Kent, K. Trout-Yakel, O. Larios, V. Allen, B. Lee, and C. Nadon. 2010. High-throughput genome sequencing of two *Listeria monocytogenes* clinical isolates during a large foodborne outbreak. *BMC Genomics* 11:120.
30. Gombas, D., Y. Chen, R. Clavero, and V. Scott. 2003. Survey of *Listeria monocytogenes* in ready-to-eat foods. *J. Food Prot.* 66:559–569.
31. Goulet, V., C. Jacquet, P. Martin, V. Vaillant, E. Laurent, and H. de Valk. 2006. Surveillance of human listeriosis in France, 2001–2003. *Euro Surveill.* 11:79–81.
32. Heisick, J., D. Wagner, M. Nierman, and J. Peeler. 1989. *Listeria* spp found on fresh-market produce. *Appl. Environ. Microbiol.* 55:1925–1927.
33. Ho, A., V. Lappi, and M. Wiedmann. 2007. Longitudinal monitoring of *Listeria monocytogenes* contamination patterns in a farmstead dairy processing facility. *J. Dairy Sci.* 90:2517–2524.
34. Hu, Y., K. Gall, A. Ho, R. Ivanek, Y. Grohn, and M. Wiedmann. 2006. Daily variability of *Listeria* contamination patterns in a cold-smoked salmon processing operation. *J. Food Prot.* 69:2123–2133.
35. LaBorde, L. F. 2006. Control of *Listeria monocytogenes* in mushroom growing and packing environments. *Mushroom News* 54(11):21–26.
36. Liu, D., and H. J. Busse. 2010. Molecular detection of food-borne pathogens. CRC Press, New York.
37. Liu, D., M. Lawrence, A. Ainsworth, and F. Austin. 2005. Comparative assessment of acid, alkali and salt tolerance in *Listeria monocytogenes* virulent and avirulent strains. *FEMS Microbiol. Lett.* 243:373–378.
38. Liu, D., M. Lawrence, M. Wiedmann, L. Gorski, R. Mandrell, A. Ainsworth, and F. Austin. 2006. *Listeria monocytogenes* subgroups IIIA, IIIB, and IIIC delineate genetically distinct populations with varied pathogenic potential. *J. Clin. Microbiol.* 44:4229–4233.
39. Nieto-Montenegro, S., J. Brown, and L. LaBorde. 2008. Development and assessment of pilot food safety educational materials and training strategies for Hispanic workers in the mushroom industry using the Health Action Model. *Food Control* 19:616–633.
40. Ojeniyi, B., H. Wegener, N. Jensen, and M. Bisgaard. 1996. *Listeria monocytogenes* in poultry and poultry products: epidemiological investigations in seven Danish abattoirs. *J. Appl. Bacteriol.* 80:395–401.
41. Olsen, S., M. Patrick, S. Hunter, V. Reddy, L. Kornstein, W. MacKenzie, K. Lane, S. Bidol, G. Stoltman, D. Frye, I. Lee, S. Hurd, T. Jones, T. LaPorte, W. Dewitt, L. Graves, M. Wiedmann, D. Schoonmaker-Bopp, A. Huang, C. Vincent, A. Bugenhagen, J. Corby, E. Carloni, M. Holcomb, R. Woron, S. Zansky, G. Dowdle, F. Smith, S. Ahrabi-Fard, A. Ong, N. Tucker, N. Hynes, and P. Mead. 2005. Multistate outbreak of *Listeria monocytogenes* infection linked to delicatessen turkey meat. *Clin. Infect. Dis.* 40:962–967.
42. Oravcova, K., T. Trncikova, T. Kuchta, and E. Kaclikova. 2008. Limitation in the detection of *Listeria monocytogenes* in food in the presence of competing *Listeria innocua*. *J. Appl. Microbiol.* 104:429–437.
43. Petran, R., and K. Swanson. 1993. Simultaneous growth of *Listeria monocytogenes* and *Listeria innocua*. *J. Food Prot.* 56:616–618.
44. Phelps, L. 2010. MGAPs: where we've been, where are we going? *Mushroom News* 59(9):4–5.
45. Ryser, E. T., and C. W. Donnelly. 2001. *Listeria*, p. 348–349. In F. P. Downes and K. Ito (ed.), *Compendium of methods for the microbiological examination of foods*. American Public Health Association, Washington, DC.
46. Samadpour, M. P., M. W. Barbour, T. Nguyen, T. M. Cao, F. Buck, G. A. Depavia, E. Mazengia, P. Yang, D. Alfi, M. Lopes, and J. D. Stopforth. 2 August 1999. Occurrence of *Listeria monocytogenes*, *Salmonella*, *Escherichia coli* O157:H7 and other Shiga-like toxin-producing *E. coli* in retail fresh ground beef, bean sprouts and mushrooms. In 86th Annu. Meet. Int. Assoc. Food Environ. Sanitarians, Dearborn, MI.
47. Seeliger, H. P. R., and D. Jones. 1986. *Listeria*, p. 1235–1245. In P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), *Bergey's Manual of systematic bacteriology*, 9th ed., vol. 2. Williams & Wilkins Co., Baltimore.
48. Sleator, R., C. Gahan, and C. Hill. 2003. A postgenomic appraisal of osmotolerance in *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 69:1–9.
49. Suslow, T., and L. Harris. 2000. Guidelines for controlling *Listeria monocytogenes* in small- to medium-scale packing and fresh-cut

- operations. Available at: <http://anrcatalog.ucdavis.edu/pdf/8015.pdf>. Accessed 12 January 2012.
50. Tamura, K., J. Dudley, M. Nei, and S. Kumar. 2007. MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 24:1596–1599.
 51. Thimothe, J., K. Nightingale, K. Gall, V. Scott, and M. Wiedmann. 2004. Tracking of *Listeria monocytogenes* in smoked fish processing plants. *J. Food Prot.* 67:328–341.
 52. U.S. Department of Agriculture. 2004. FSIS rule designed to reduce *Listeria monocytogenes* in ready-to-eat meat and poultry. Available at: http://www.fsis.usda.gov/Fact_Sheets/FSIS_Rule_Designed_to_Reduce_Listeria/index.asp. Accessed 12 January 2012.
 53. U.S. Food and Drug Administration. 2006. Monterey Mushrooms recalls fresh sliced white and baby bella mushrooms in PA, MD, NC, NJ, NY, OH, and VA because of possible health risk. Available at: http://www.fda.gov/oc/po/firmrecalls/monterey09_08.html. Accessed 25 January 2012.
 54. U.S. Food and Drug Administration. 2008. Guidance for industry: control of *Listeria monocytogenes* in refrigerated or frozen ready-to-eat foods; draft guidance. Available at: <http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/FoodProcessingHACCP/ucm073110.htm>. Accessed 25 January 2012.
 55. U.S. Food and Drug Administration. 2011. Environmental assessment: factors potentially contributing to the contamination of fresh whole cantaloupe implicated in a multi-state outbreak of listeriosis, October 19, 2011. Available at: <http://www.fda.gov/Food/FoodSafety/FoodborneIllness/ucm276247.htm>. Accessed 25 January 2012.
 56. Vannetten, P., I. Perales, A. Vandemoosdijk, G. Curtis, and D. Mossel. 1989. Liquid and solid selective differential media for the detection and enumeration of *L. monocytogenes* and other *Listeria* spp. *Int. J. Food Microbiol.* 8:299–316.
 57. Vazquez-Boland, J., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland, and J. Kreft. 2001. *Listeria* pathogenesis and molecular virulence determinants. *Clin. Microbiol. Rev.* 14:584–640.
 58. Ward, T., T. Ducey, T. Usgaard, K. Dunn, and J. Bielawski. 2008. Multilocus genotyping assays for single nucleotide polymorphism-based subtyping of *Listeria monocytogenes* isolates. *Appl. Environ. Microbiol.* 74:7629–7642.
 59. Wiedmann, M., J. Bruce, C. Keating, A. Johnson, P. McDonough, and C. Batt. 1997. Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. *Infect. Immun.* 65:2707–2716.
 60. Zhang, W., B. Jayarao, and S. Knabel. 2004. Multi-virulence-locus sequence typing of *Listeria monocytogenes*. *Appl. Environ. Microbiol.* 70:913–920.
 61. Zhang, W., and S. Knabel. 2005. Multiplex PCR assay simplifies serotyping and sequence typing of *Listeria monocytogenes* associated with human outbreaks. *J. Food Prot.* 68:1907–1910.
 62. Zitz, U., M. Zunabovic, K. Domig, P. Wilrich, and W. Kneifel. 2011. Reduced detectability of *Listeria monocytogenes* in the presence of *Listeria innocua*. *J. Food Prot.* 74:1282–1287.