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, *pfkA*, *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, 2/2c, 3a, 3b, 3c, 4a, 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 (LIlll) 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
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 (≤4°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 m^2) 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
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 steam treated 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-β-glucose, and methyl-α-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 Brilliant 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 (1,9, 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 Facilitya

<table>
<thead>
<tr>
<th>Zone</th>
<th>No. of samples</th>
<th>No. of isolates</th>
<th>Lm</th>
<th>Lin</th>
<th>Lw</th>
<th>Lg</th>
<th>Ls</th>
<th>Liv</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>20</td>
<td>3</td>
<td>13</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>3</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>2</td>
<td>0</td>
<td>2</td>
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<td>50</td>
<td>4</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>14</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>184</td>
<td>29</td>
<td>3</td>
<td>19</td>
<td>6</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

\[ a \] Lm, L. monocytogenes; Lin, L. innocua; Lw, L. welshimeri; Lg, L. grayi; Ls, L. seeligeri; Liv, L. ivanovii.

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 MTDF 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 I A9, phase I B9, and phase I 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.

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 MTDF 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 were 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.
(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 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 [LIPI-1], inlAB locus, bsh, and hpt). They also share gene deletions similar to those of L. innocua (e.g., inIC, inII, inIJ, the internalin cluster between ascB and dapE, and the arginine deiminase island lmo0036-lmo0041) (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 (bilB 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 MTDF may be conducive to L. monocytogenes growth and that sanitation practices should be reviewed. A policy limiting movement of equipment and personnel between MTDF 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 lost many virulence genes. Although the MTDF 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 MTDF 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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