THERMAL SANITIZING TREATMENTS FOR ELIMINATING \textit{LISTERIA MONOCYTOGENES} FROM INDUSTRIAL MUSHROOM DISC SLICING EQUIPMENT

A Thesis in Food Science by Hilary M. Tobin

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ABSTRACT

*Listeria monocytogenes* can colonize processing environments and transfer to food contact equipment. Current industrial mushroom slicers consist of successive cutting disks aligned and separated by spacers. This design makes complete disassembly for cleaning and sanitizing time consuming and therefore, impractical. Although current industry practice is to remove the slicer head for submersion in hot water, this practice must be validated for elimination of *L. monocytogenes*. In this study, effective thermal sanitization treatment parameters for mushroom disk slicers were determined.

A 6-strain *L. monocytogenes* cocktail was prepared from the most heat tolerant strains isolated from mushrooms, environmental samples, and non-mushroom associated outbreaks. The cocktail was used to determine the effect of incubation time on heat tolerance of *L. monocytogenes* in planktonic and surface adhered forms. Planktonic cells heat treated immediately after inoculation were the most heat tolerant growth state and were used to study thermal destruction kinetics of *L. monocytogenes* and *L. innocua*. Thermal death time curves were generated by immersing samples in a circulating water bath at 50, 60, and 70°C for up to 150 minutes and D values were calculated for 50, 60, and 70°C. *L. innocua* had equal or greater heat tolerance compared to *L. monocytogenes* cells and was therefore a suitable non-pathogenic surrogate for use in subsequent pilot plant thermal sanitization validation studies.

Heat penetration into the mushroom slicer during immersion in a temperature-controlled, recirculating, clean-out-of-place (COP) wash tank filled with water heated to 55, 65, and 75°C was studied using embedded thermocouples and a data logger. A similar study was performed using moist air heated at 85°C. Spacers on the top axle heated slightly more slowly than those on
the bottom axle. Therefore, the inner circumference of the spacer on the top axle was used as the inoculation site in the subsequent pilot plant validation study.

The achievable inoculum concentration on the inner circumference and the previously calculated D-values at 50, 60, and 70°C were used to calculate times required to achieve at least 7 log reductions of *L. innocua* at tank water temperatures of 55, 65, and 75°C. After each treatment temperature and time combination, *L. innocua* was undetectable after enrichment. The same results can be achieved using moist air heated to 85°C. This thermal sanitization validation study can therefore be used to provide practical recommendations to the mushroom industry for eliminating *L. monocytogenes* on mushroom slicers.
# TABLE OF CONTENTS

LIST OF FIGURES .................................................................................................................. vii  
LIST OF TABLES ................................................................................................................... viii  
ACKNOWLEDGEMENTS ........................................................................................................ ix  
CHAPTER 1. STATEMENT OF THE PROBLEM ................................................................. 1  
1.1 Statement of the problem ......................................................................................... 1  
1.2 Research objectives ............................................................................................... 2  
CHAPTER 2. LITERATURE REVIEW ............................................................................. 3  
2.1 Listeriosis and *Listeria monocytogenes* ................................................................ 3  
2.1.1 Listeriosis ........................................................................................................ 3  
2.1.2 Characteristics of *Listeria monocytogenes* ...................................................... 4  
2.1.3 Retail surveys of fresh produce ........................................................................ 6  
2.1.4 Recent outbreaks attributed to *Listeria monocytogenes* in fresh produce .... 7  
2.1.5 Outbreak Surveillance ....................................................................................... 8  
2.1.6 Recalls attributed to *Listeria monocytogenes* in fresh mushrooms .............. 9  
2.2 The mushroom growing, packing, and slicing process and potential sources and routes of transmission and growth of *Listeria* spp. ................................................. 10  
2.3 Principles and standards for sanitary design .......................................................... 12  
2.2.1 Outbreaks due to poor sanitation and sanitary design .................................... 12  
2.2.2 Sanitary design characteristics ....................................................................... 13  
2.2.3 Government regulations and guidance .............................................................. 15  
2.2.4 Non-government organizations ...................................................................... 17  
2.2.5 Current industrial mushroom slicing equipment ............................................ 18  
2.4 Validation of sanitation practices ......................................................................... 19  
CHAPTER 3 .......................................................................................................................... 22  
Abstract .............................................................................................................................. 22  
3.1 Introduction .............................................................................................................. 23  
3.2 Materials and Methods .......................................................................................... 27  
3.2.1 Thermal destruction characteristics of planktonic and surface attached *L. monocytogenes* and identification of a *L. innocua* strain as a suitable surrogate organism .............................................................................................................................. 27  
3.2.1.1. Selection of strains and preparation of working cultures .......................... 27  
3.2.1.2. Screening study of *Listeria* spp. strains for heat tolerance and preparation of a multi-strain *L. monocytogenes* cocktail .......................................................... 28  
3.2.1.3. Heat tolerance of planktonic vs. adhered cells *L. monocytogenes* ........ 29  
3.2.1.4. *Listeria* spp. thermal destruction kinetic study ..................................... 30  
3.2.2 Heat penetration study on a commercial mushroom slicer head ..................... 31  
3.2.2.1. Equipment used ....................................................................................... 31  
3.2.2.2. Slicer head heat penetration experiments ............................................. 32
3.2.3. Food safety pilot plant challenge study .................................................. 35
3.2.4. Statistical analysis .................................................................................. 37
3.3 Results and Discussion .............................................................................. 37
3.3.1. Screening study of *Listeria* spp. strains for heat tolerance and preparation of a multi-strain *L. monocytogenes* cocktail .......................................................... 37
3.3.2. Heat tolerance of planktonic vs. adhered cells *L. monocytogenes* .......... 38
3.3.3. *Listeria* spp. Thermal destruction kinetic study ..................................... 39
3.3.4 Heat penetration study on a commercial mushroom slicer .................... 40
3.3.5. Food safety pilot plant challenge study .................................................. 43
3.4 Conclusions .............................................................................................. 44

CHAPTER 4. SUMMARY AND RECOMMENDATIONS TO INDUSTRY ............ 59
4.1 Summary .................................................................................................... 59
4.2 Recommendations to Industry ................................................................. 60

REFERENCES ................................................................................................ 65
LIST OF FIGURES

Figure 3.1. Slicer head of a 3-lane mushroom disk slicer head. ........................................ 53

Figure 3.2 Diagram of the spacer and blade orientation (not drawn to scale) and insertion orientation of thermocouple wire. ................................................................. 53

Figure 3.3. Spatial orientation of thermocouples for COP tank temperature distribution experiment................................................................. 54

Figure 3.4a. Heat penetration curves for outer and inner circumference locations at spacers positioned on the top or bottom slicer blade axles during immersion of the slicer head in water at 55°C. ........................................................................................................................................ 55

Figure 3.4b. Heat penetration curves for outer and inner circumference locations at spacers positioned on the top or bottom slicer blade axles during immersion of the slicer head in water at 65°C ........................................................................................................................................ 56

Figure 3.4c. Heat penetration curves for outer and inner circumference locations at spacers positioned on the top or bottom slicer blade axles during immersion of the slicer head in water at 75°C. ........................................................................................................................................ 57

Figure 3.5. Heat penetration curves for outer and inner circumference locations at spacers positioned on the top or bottom slicer blade axles during immersion of the slicer head in moist air at 85°C ........................................................................................................................................ 58
LIST OF TABLES

Table 3.1. *Listeria* spp. strains selected for heat tolerance screening, population decreases after heating at 60°C for 6 minutes, and strains selected for inclusion in a 6-strain *L. monocytogenes* cocktail................................................................. 45

Table 3.2. Decreases in planktonic and stainless steel adhered *L. monocytogenes* cell populations in samples incubated at 30°C for up to 7 days prior to heating at 60°C for 6 minutes..... 46

Table 3.3. Populations of *L. monocytogenes* and *L. innocua* after treatments at 50, 60, and 70°C ................................................................................................................................. 47

Table 3.4. D values for *L. monocytogenes* and *L. innocua* determined at 50°, 60°, and 70°C and calculated z values .................................................................................................................. 48

Table 3.5. Time to reach target temperature of outer and in circumference of spacers when immersed in hot water................................................................. 49

Table 3.6. Time to reach target temperature of outer and in circumference of spacers when immersed in heated moist air ................................................................. 50

Table 3.7. Recovery of *L. innocua* from inner spacer circumference surface immediately after air drying; and after drying, assembly, immersion in water, and disassembly .......... 51

Table 3.8. Microbial validation study results ................................................................. 52

Table 4.1. Recommended heating parameters to achieve an equivalent reduction of reductions in *L. monocytogenes* of at least 7 logs ................................................................. 65
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CHAPTER 1. STATEMENT OF THE PROBLEM

1.1 Statement of the Problem

A recent microbial survey of a mushroom processing facility reported that 18.8% of samples taken from non-food contact surfaces were positive for *Listeria monocytogenes*. The pathogen was primarily found in continuously wet washing, slicing, and packaging areas. Current industrial mushroom slicers do not fully conform to good sanitary design standards. There are many metal-to-metal junctions that occur between blades and spacers, which make penetration of cleaning and sanitizing solutions difficult. Additionally, frequent and complete disassembly for cleaning and sanitizing is time consuming and often impractical given the demands for continuous production. Therefore, there is a risk for slicing equipment and sliced mushrooms to become contaminated. The current practice for deep cleaning is to boil the detachable slicer head in water for up to 1 hour between complete disassembly. However, thermal sanitization processes have not been validated to ensure *L. monocytogenes* is reduced to a safe level. In addition, accelerated slicer corrosion and loss of lubricant oils have been reported. Under the Food Safety Modernization Act of 2011, the Food and Drug Administration requires food-processing facilities to provide evidence that the equipment they use can be effectively cleaned and sanitized. Therefore, the mushroom industry will need to assure that mushroom slicers will not become a source of contamination.
1.2 Research Objectives

The broad objective of this research is to determine the heating time/temperature parameters needed to eliminate *L. monocytogenes* throughout an industrial mushroom disk slicer head during a hot water treatment in a clean-out-of-place (COP) tank. Specific objectives are:

1. *To perform thermal destruction studies at 50, 60, and 70°C.*

    The times needed to reach at least a 7-log reduction of *L. monocytogenes* and *L. innocua*, intended for use as a surrogate, will be determined for use in subsequent validation studies.

2. *To perform a heat penetration study on a mushroom disk slicer head to determine the location of cold spots during thermal sanitizing in a clean-out-of-place (COP) tank, as well as the time to reach target temperatures at these locations.*

    Cold spots represent the most conservative approach for inoculation sites in subsequent validation studies. Times to reach target temperatures will be used to develop subsequent recommendations to industry.

3. *To validate time/temperature parameters needed to achieve at least a 7-log reduction of *L. monocytogenes* at cold spots within the mushroom disc slicer head during thermal sanitizing in COP tank.*

    A *L. innocua* surrogate with similar thermal destruction characteristics as *L. monocytogenes*, identified in the first objective, will be used to inoculate the previously-identified cold spots in the second objective. Thermal destruction will be monitored during the COP treatment needed for at least a 7-log reduction as previously determined in the first objective.
CHAPTER 2. LITERATURE REVIEW

2.1 Listeriosis and *Listeria monocytogenes*

2.1.1 Listeriosis

The first case of *Listeria monocytogenes* infection occurred in 1924 and was reported by Murray et al. (1926) and Goebel et al. (2001) when six instances of sudden death were observed in young rabbits bred for laboratory testing. After isolating Gram-positive bacilli from a pregnant rabbit, the researchers concluded that the causative organism had not been previously described. The microorganism was named *Bacterium monocytogenes* because its primary characteristic was causing the production of large mononuclear leukocytes. Although initially thought to be an animal pathogen, in 1979, it was isolated in raw vegetables, including celery, tomatoes, and lettuce, and thought to have been the cause of a listeriosis outbreak that sickened at least 23 people in the Boston area (Ho et al. 1986). In 1981, *L. monocytogenes* contaminated cabbage that had been stored in a barn where sheep manure was kept and implicated in a listeriosis outbreak traced to coleslaw (Schlech et al. 1983).

Cases of listeriosis occur after one has ingested food contaminated with *L. monocytogenes*. Generally, at least 1000 CFU/g is necessary to cause illness. The incubation period for listeriosis ranges from 1 to 70 days, with an average of approximately 3 weeks (Luber et al. 2010). Infection occurs when *L. monocytogenes* crosses the mucosal barrier of the gut through endocytosis whereupon it enters the bloodstream. It has the ability to function as an intracellular organism through entrance into the cytoplasm, where it divides, and sequentially moves from one cell to the next, evading antibodies. Infection symptoms include stiff neck,
movement disorders, seizures, meningitis, brainstem encephalitis, brain abscess, endocarditis, gastroenteritis, and other health issues (Goldfine and Shen 2007).

The CDC has estimated that L. monocytogenes causes approximately 1,600 illnesses, 1,500 hospitalizations, and 260 deaths annually (USDA 2014). Listeriosis accounts for only 0.02% of all foodborne illness, causing 1 to 9 cases per million people each year; however, it accounts for 28% of deaths resulting from foodborne illness (Tompkin 2002). The fatality rate of listeriosis is 20-30%, as compared with 0.5% for Salmonella or E. coli O157:H7. Cases not resulting in death include symptoms such as gastroenteritis, fever, or miscarriage, which may be under-diagnosed (Scallan et al. 2011). The most susceptible groups are the elderly, persons with a lowered immunity, such as AIDS patients, organ transplant recipients, cancer patients, pregnant women, and children with immune systems that are not fully developed (Luber 2010).

2.1.2 Characteristics of Listeria monocytogenes

L. monocytogenes is a psychrotrophic, facultative, anaerobic Gram-positive bacterium (Gandhi and Chikindas 2007). It exists naturally as a saprophyte in environments such as soil, water, and decaying vegetation. It can also exist as a pathogen in animals and humans. (Vazquez-Boland et al. 2001, Goebel et al. 2001, Ferreira 2014, Wen et al. 2009).

The ability of L. monocytogenes to colonize, grow, and persist on food-processing surfaces and equipment, allows it to survive for extended periods in food processing facilities (Ryser 2007). Generally, if an area remains wet for longer than six hours, the environment allows L. monocytogenes to grow (UFPA 2013). Although the optimal growing temperature of L. monocytogenes is 30-37°C, it can also survive and grow at refrigeration temperatures of 4-10°C (Luber et al. 2011, Goldfine and Shen 2007).
*L. monocytogenes* is sensitive to heat; temperatures as low as 75°C for 10 seconds have been effective in killing the bacterium (UFPA 2013). Although heat treatments applied to ready-to-eat products are not often practical, heat can be used to effectively control *L. monocytogenes* on food contact surfaces and equipment (UFPA 2013).

*L. monocytogenes* can exist as planktonic cells suspended in a liquid or can adhere to solid surfaces where they can ultimately develop into a biofilm. Biofilms are defined as a “microbial-derived sessile community, where cells are irreversibly attached to a substratum, interface, or to each other, and are embedded in a matrix of extracellular polymeric substrate” (Donlan and Costerton 2002). Surface adherence and biofilm formation can be attributed to physiochemical interactions between bacteria and surfaces, such as charge, hydrophobicity, Van Der Waals forces, and acid-base interactions (Trinetta et al. 2012). Initial attachment of cells is further influenced by surface heterogeneity, the presence of conditioning films consisting of organic soils, and presence of surface irregularities such as pits, cracks, rough welds, and crevices (Chmielewski and Frank 2003). Cells in biofilms have been shown to have a higher tolerance to sanitizers, antimicrobials, and heat (Costerton et al. 1999, LeChevallier et al. 1998, Frank and Koffi 1990, Lee and Frank, 1991, Trinetta et al. 2012). However, the mechanism for increased heat resistance of *Listeria* within biofilms is not known (Chmielewski and Frank 2003).

Growth conditions and age of culture can affect heat tolerance of a culture. Frank and Koffi (1990) compared heat tolerance of planktonic, versus adhered cells, on glass slides and reported a significantly smaller decrease in adhered cells compared to planktonic when exposed to 55 and 70°C for 5 minutes (1990). Furthermore, Oh and Marshall (1995) reported that 1-day adhered cells on stainless steel were more sensitive to heat treatment than planktonic cells. Older
adhered populations (7 days) were slightly more heat tolerant than planktonic cells. However, another study by Lee and Frank (1991) found that when stainless steel slides containing adhered cells for either 4 hours or 8 days were heated at 72°C, both populations were equally inactivated after 1 minute. Therefore, these results make it necessary to test heat resistance of varying ages of planktonic and adhered cells.

2.1.3 Retail surveys of fresh produce

Historically, *L. monocytogenes* has predominantly been known as a contaminant of raw milk, soft cheeses, and ready-to-eat meat products (Ferreira et al. 2014). However, the outbreak of listeriosis in Canada attributed to contaminated cabbage used to produce coleslaw (Schlech et al. 1983) led to surveys of fresh vegetables in supermarkets. Retail surveys have reported *L. monocytogenes* in cabbage, cucumbers, potatoes, radishes, broccoli, leafy greens, and tomatoes (Beuchat 1996, Harris et al. 2003, Heisick et al. 1989).

Contamination of mushrooms with *L. monocytogenes* has also been shown to occur. A retail survey of *Agaricus bisporus* mushrooms in the Pacific Northwest found that 1% of retail samples tested positive for *L. monocytogenes* (Heisick et al. 1989). *L. monocytogenes* was found in 10% and 11% of mushrooms sampled from grocery stores in the Netherlands and Minnesota, respectively (Heisick et al. 1989, Van Netten 1989). More recently, Samadpour et al. (2006) reported that 1% of 100 retail mushroom samples taken over a period of 12 months tested positive for *L. monocytogenes*. 
2.1.4 Recent outbreaks attributed to *Listeria monocytogenes* in fresh produce

A number of deadly outbreaks attributed to *L. monocytogenes* in fresh produce have occurred in the past five years. In 2010, fresh-cut celery manufactured by Sangar Fresh Cut Produce in Texas tested positive for *L. monocytogenes*, which resulted in 4 deaths (Gaul et al. 2013, UFPA 2013). In 2011, after a collaborative investigation by local, state, and federal public health and regulatory agencies, *L. monocytogenes*-contaminated whole cantaloupes produced by Jensen Farms in Colorado were found to have sickened 147 individuals across 28 states and caused 33 deaths (CDC 2011, UFPA 2013). FDA reported that the a number of factors could have contributed to the introduction spread, and growth of *L. monocytogenes*, including: (1) presence of the pathogen in the field where the cantaloupe were grown; (2) a contaminated truck which transported product to the packaging facility from the field; (3) collection of water on the packing facility floor near walkways and equipment and (4) floors and equipment which were not easily cleaned and sanitized; (6) the washing and drying equipment was previously used for raw potatoes; (7) no pre-cooling step was used to remove field heat from the cantaloupes before refrigerated storage, which promoted condensation (FDA 2012, UFPA 2013).

In 2014, five cases of listeriosis were reported, resulting in 2 deaths. A follow-up investigation showed that mung bean sprouts produced by Wholesome Soy Products Inc. in Chicago, Illinois were the source of contamination. Environmental swabs, product samples, and irrigation water samples all tested positive for *L. monocytogenes*, indicating the bacterium was not being controlled throughout the process (CDC 2014, FDA 2015, UFPA 2013).

Another listeriosis outbreak occurred in 2014, when 25 people from 12 states were infected with *L. monocytogenes* resulting in three deaths and one miscarriage. The common food consumed by the victims was prepackaged whole caramel apples produced by a number of
brands that sourced their apples from Bidart Bros. in California (CDC 2015, FDA 2015, UFPA 2013). It was hypothesized that when the sticks used for holding the apple punctured contaminated apples, small amounts of apple juice spread over the surface of the otherwise dry outer skin. The caramel coating then locked in the juice, creating an environment in which \textit{L. monocytogenes} could grow (Andrews 2015).

Most recently, in 2015, 19 people across 9 states were infected with \textit{L. monocytogenes} after consuming packaged salad produced by Dole in Springfield, Ohio. Contaminated products included kale, spinach, coleslaw, iceberg lettuce, romaine lettuce, and a number of salad blends (CDC 2016, FDA 2016, Beach 2016).

2.1.5 Outbreak Surveillance

In recent years, the number of \textit{L. monocytogenes} outbreaks that have occurred has increased; however, the increase may be in large part due to improved surveillance and detection of outbreaks by CDC and other health departments (Andrews 2015). The U.S. Centers for Disease Control and Prevention (CDC) reported only 5 \textit{L. monocytogenes} outbreaks from 1983 to 1997. In 1998, CDC introduced PulseNet, a surveillance system network shared by health departments throughout the U.S and which can quickly identify and respond to foodborne disease outbreaks. After PulseNet was implemented, an average of 2.3 listeriosis outbreaks were reported each year from 1998 to 2003. In 2004, CDC introduced an enhanced surveillance system called the “Listeria initiative” and average number of outbreaks detected per year increased to 2.9. Whole genome sequencing, which more accurately identifies pathogens compared to other system, was widely adopted by health departments in 2014 and may be at
least partially responsible for further increase in reported cases of listeriosis to 9 outbreaks per year.

2.1.6 Recalls attributed to *Listeria monocytogenes* in fresh mushrooms

To date, there have been no reported outbreaks of listeriosis associated with the consumption of fresh mushrooms. However, contamination of fresh mushrooms has been shown to be possible. Chikthimmah et al. (2007) reported growth of *L. monocytogenes* on sliced, but not whole, mushrooms at 12°C. Likewise, growth of *L. monocytogenes* on sliced mushrooms at 15°C was reported by Yuk et al. Furthermore, Gonzalez et al. (2001) and Leong et al. (2013) found growth of *L. monocytogenes* on whole mushrooms at 4-15°C was possible.

More recently, periodic testing by government agencies has resulted in several recalls. The Georgia Department of Agriculture and the Ohio Department of Agriculture issued recalls in 2003 and 2006, respectively for sliced mushrooms that tested positive for *L. monocytogenes* (FDA 2003, FDA 2006). Between 2008 and 2016, the Canadian Food Inspection Agency (CFIA) issued 6 recalls of various brands of fresh sliced mushrooms (CFIA 2008, 2011, 2012, 2014, 2015, and 2016). Furthermore, whole Enoki mushrooms (*Flammulina velutipes*) produced in Pennsylvania were recalled in 2009 after *L. monocytogenes* contamination was detected (FDA 2009).
2.2 The mushroom growing, packing, and slicing process and potential sources and routes of transmission and growth of Listeria spp.

In the first stage of mushroom production, known as Phase 1 composting, a growth substrate is prepared from raw materials, including horse and chicken manure, hay, and other organic materials, which are mixed, wetted, and piled into long rows where fermentation occurs for 6 to 14 days (Royse and Beelman 2007). Although temperatures as high as 180°F are reached within the interior of the rows, this process does not completely eliminate pathogens that might occur in the raw materials. This is evidenced by results from a survey of a small-scale mushroom production facility where L. monocytogenes was present in the area used for phase I composting (Viswanath et al. 2012).

Next, indoor phase II composting incorporates a pasteurization step that kills mesophilic bacteria, insects, nematodes, fungi, or other pests that may be present in the compost. Weil et al. (2013) reported phase II composting can achieve at least a 7-log reduction and is an effective control measure for eliminating L. monocytogenes.

Agaricus bisporus-inoculated grain, known as mushroom spawn, is then mixed into the compost and allowed to grow. Casing soil, a mixture of peat moss and ground limestone, is applied to the top of the compost (Royse and Beelman 2007). In contrast to the compost, the casing material is not pasteurized and therefore contains high populations of indigenous microorganisms. Studies by Chikthimmah (2006) and O’Patchen (2011) reported 3 to 4-log reductions in L. monocytogenes and Salmonella spp. in un-heated casing soil while previously autoclaved soil resulted in no reductions. The authors suggested that casing soil may provide unfavorable conditions for pathogens and thus can be considered a food safety control measure.
During the growth period, mushrooms are watered regularly. This step is unlikely to introduce pathogens as long as the water is tested regularly to assure absence of pathogens.

Mushrooms are harvested by hand, which may be a potential route of contamination; however training of personal hygiene and safe food-handling practices is regularly emphasized as part of the industry’s Mushroom Good Agricultural Practices (MGAP) program (MGAP 2011). Harvested mushrooms are then packed into containers and loaded onto trucks for transportation to packaging and slicing facilities.

Upon receipt at the processing facility, mushrooms are vacuum-cooled, and then packaged either whole or sliced. Mushrooms for slicing may be either washed or unwashed prior to cutting. The mushrooms are transferred to the slicer by a specialized conveyor with V-shaped aisles, which aligns the mushrooms in the correct orientation before slicing with stainless steel disk blades. Workers weigh and fill the sliced mushrooms into polystyrene trays and over-wrap them with perforated plastic film. The slicing environment may be a source of *L. monocytogenes* contamination. Murugesan et al. (2015) performed a survey of 255 samples of non-food contact surfaces in a mushroom processing facility over 13 months. Results showed an incidence rate of 18.8% for *L. monocytogenes*, which was primarily found in continuously wet washing, slicing, and packaging areas. The study also demonstrated that improvements in basic sanitation procedures can lead to reduced incidence of *L. monocytogenes* in the processing facility.

Several studies have shown an increased risk for product contamination when non-food contact surfaces are contaminated. Thimothe et al. (2004) reported the prevalence of *L. monocytogenes* in smoked fish processing plants had a statistically significant positive relationship with the prevalence of *L. monocytogenes* in finished product samples. Berrang et al. (2010) reported that a subtype of *L. monocytogenes* in raw chicken was identical to a persistent
subtype recovered in a drain in the processing facility. Therefore, the results reported by Murugesan et al. (2015) are concerning since *Listeria* contamination on non-food contact surfaces was relatively high.

Slicing equipment, in particular, is a concern due to the manner in which it was designed. If contaminated and insufficiently cleaned and sanitized over time, the slicing equipment has the potential to become a reservoir for *L. monocytogenes* that can continuously contaminate mushrooms.

2.3 Principles and standards for sanitary design

2.2.1 Outbreaks due to poor sanitation and sanitary design

In recent years, poor sanitation practices and equipment design have been identified as the cause for a number of food safety outbreaks. In 1991, *Salmonella enteritidis*-contaminated ice cream produced by Schwan’s Inc. caused a nationwide outbreak, during which 80 confirmed cases of salmonellosis occurred. Inadequate record keeping of sanitation procedures, lack of routine inspections, the practice of hauling the ice cream premix in truck previously used to transport non-pasteurized ingredients, and cracks in the interior of the tanker truck may have contributed to the cause (Hennessy et al. 1996, Schmidt 2013). In 2008, contamination of Maple Leaf Foods’ deli meats caused a *L. monocytogenes* outbreak in Canada, which resulted in 422 illnesses and 57 deaths. A follow-up investigation indicated that Maple Leaf Foods management was aware of *L. monocytogenes* contamination within their plant, but did not conduct a trend analysis as part of a *Listeria* control policy. Additionally, the poor sanitary design of the meat slicers made cleaning and sanitizing difficult, thereby allowing niche sites for *L. monocytogenes* to grow. The company reported that slicer disassembly, thorough cleaning and sanitization, and
then reassembly necessitated shutting down the plant for three days (CFIA 2009). In 2009, a *Salmonella Typhimurium* outbreak was caused by contaminated peanuts and peanut products. It was thought that contamination was due to poor facility maintenance, poor equipment design and maintenance, and inadequate cleaning and sanitizing programs. There were 714 cases of infection across 46 states, 9 of which contributed to patients’ deaths (CDC 2009). Lastly, poor equipment design, construction, cleaning, and maintenance were thought to be a major cause of the previously mentioned *L. monocytogenes* outbreak associated with cantaloupes that resulted in 33 deaths (CDC 2012).

### 2.2.2 Sanitary design characteristics

The U.S. Food and Drug Administration (FDA) has reported that cross contamination and improper cleaning and sanitation are among the biggest risk factors contributing to foodborne pathogen contamination, spread, and growth (FDA National Retail Food Team 2009). More than 80% of existing cleaning and sanitizing processes in the food industry are not validated and are poorly documented. This finding is likely one of the root causes of food safety recalls and outbreaks (Timmerman 2013).

The main goal of good sanitary design is to make it easier to clean and sanitize equipment in order to reduce the potential for contamination by biological, chemical, and physical hazards (21 Code of Federal Regulations (CFR) Part 117, 3-A 2016, AMI 2014, EHEDG 2004, IFPA 2003, Schmidt 2013). *L. monocytogenes* can attach to and grow on equipment surfaces and form biofilms, especially on surfaces that are not adequately cleaned and sanitized (Chmielewski and Frank 2007). Food processors want cleaning and sanitizing to be easy to conduct, in the shortest
time possible, and at the lowest cost possible, in terms of expenses related to purchasing chemicals, energy inputs, and labor costs (Fryer et al. 2013).

Good sanitary design principles are especially important for food contact equipment with surfaces where food residue can drip, drain, diffuse, be drawn, or come in direct contact with, or nonfood contact surfaces (21 CFR Part 110). Sanitation practices should cause little damage to the equipment through exposure to high temperatures or corrosive chemicals, should be safe for employees to conduct, should produce as little waste as possible, and be effective for reducing harmful microorganisms to safe levels (Lelieveld 1985). Materials used for food contact surfaces should be “smooth, impervious, nontoxic, nonabsorbent, and corrosion resistant under conditions of intended use” and polished to a smooth finish (AMI 2014, 3-A 2016, EHEDG 2004, Schmidt 2013). Stainless steel is generally preferred, as it is nontoxic and corrosion-resistant when in contact with food (21 CFR Part 117, 3-A 2016, AMI 2014, EHEDG 2004), but other metal and nonmetal materials, such as plastics, rubber, and ceramics can be acceptable. (AMI 2014, 3-A 2016, EHEDG 2004, Schmidt 2013, UFPA 2013). Furthermore, equipment must be free of cracks, crevices, or sharp interior angles to prevent dead space for food, water, and, therefore, biological, chemical, and physical hazards to reside (21 CFR Part 117, AMI 2014, 3-A 2016, UFPA 2013). Equipment must also be installed, operated, and maintained properly to ensure effective cleaning and prevent cross contamination (3-A 2016, EHEDG 2004, Schmidt 2013). Equipment must be accessible for inspection, maintenance, cleaning and sanitizing, the procedures of which must be clearly written, designed, and proven effective and efficient (AMI 2014, UFPA 2013).

Lelieveld (2015) added to the concept of sanitary design by stressing the need for proper training of staff. It was recommended that manufacturers of equipment for the food
industry should have knowledge of hygienic design and the basics of microbiology. Additionally, food handlers should know that poorly designed and incorrectly used equipment could result in microbial contamination. Furthermore, food safety inspectors should be adequately trained to determine if equipment follows good sanitary design. Design of equipment should be a collaborative effort between equipment manufacturers and food processors to prevent foodborne illness by contamination of food from equipment (Lelieveld 2015).

The first step in achieving these goals is good sanitary equipment design. The second step is validating that sanitization practices are effective under normal food processing conditions.

2.2.3 Government regulations and guidance

There are many common standards and recommendations for sanitary design developed by government and industry organizations. Outside the United States, laws and regulations that address sanitary design and cleaning and sanitizing practices include Cleaning and Validation Guidelines in Canada (Canada HPFB 2005, Timmerman 2013), Law on Food and Feed and subordinated regulations in Germany (Timmerman 2013, USDA FAS 2015), Regulations and the Machinery Directive in Europe (CEN 2009, Timmerman 2013), and the Codex Alimentarius, which provides voluntary international sanitation standards (FAO 2003, Timmerman 2013).

In the U.S., the Department of Agriculture (USDA) Food Safety & Inspection Service (FSIS; USDA FSIS 2016) provides guidelines for equipment used in meat, poultry, and egg product facilities. The USDA Agricultural Marketing Service (AMS) (USDA AMS 2016) also has an approval process for dairy equipment. The Food and Drug Administration (FDA) addresses equipment fabrication and cleanability in its Current Good Manufacturing Practice in Manufacturing, Packaging, and Holding Human Food (cGMPs) (21 CFR Part 110). As directed in the Food Safety Modernization Act of 2011, GMP standards will be updated in 2018 through
the recent Current Good Manufacturing Practice, Hazard Analysis, and Risk-Based Preventive Controls for Human Foods regulation (21 CFR Part 117).

The Interagency Retail *L. monocytogenes* Risk Assessment (USDA 2014) was jointly developed by FSIS and FDA, in consultation with Center for Disease Control and Prevention (CDC) to minimize the occurrence of listeriosis attributed to products from retail markets in the U.S. The risk assessment focused on observations of deli employees’ work routines, potential for contamination from incoming products, and the ability of *L. monocytogenes* to spread from a slicer to food and included a dose-response model (FSIS 2015). A key finding of the risk assessment was that pre-slicing ready-to-eat products in retail delis increases the predicted risk of *L. monocytogenes* infection by 6%. A further finding was that equipment used to handle, prepare, and store ready-to-eat products should be cleaned at least every 4 hours, a standard that is incorporated in the 2013 FDA Food Code (FDA 2013) (FSIS 2015).

More recently, the United States Congress enacted the Food Safety Modernization Act of 2011 (FSMA) (FSMA 2011), under which FDA wrote the regulation “Current Good Manufacturing Practice and Hazard Analysis and Risk-Based Preventive Controls for Human Food” (21 CFR Part 117). The regulation requires specific new requirements for developing and documenting validated preventive control measures that go far beyond the general requirements established earlier in 21 CFR Part 110. In particular, FSMA mandates science-based validation of preventive controls, including those for cleaning and sanitizing of food contact equipment (Schmidt 2013). Under this regulation, facilities that slice mushrooms will need to provide evidence that the equipment they use can be effectively cleaned and sanitized so that it will not become a source of contamination.
2.2.4 Non-government organizations

Nongovernmental organizations, including 3-A Sanitary Standards Inc. (3-A SS) (3-A Sanitary Standards 2016), European Hygienic Design Group (EHEDG) (EHEDG 2004), and National Sanitation Foundation (NSF) (NSF 2016) International have also established standards and recommendations that stress the importance of hygienic design and sanitation practices (Schmidt and Erickson 2005).

3-A SS was originally developed for use in the dairy and egg industries (Schmidt 2013). 3-A SS works with other industry groups to improve standards, and they are active in training, workshops, and sharing knowledge. They provide assurance by conducting on-site evaluations of equipment based on general principles of hygienic design, construction, fabrication, installation, operation, and maintenance (3-A Sanitary Standards 2016, Schmidt 2013).

The European Hygienic Design Group (EHDG) is a consortium of equipment manufacturers, food industries, research institutes, and public health authorities that provide standards for processing equipment construction and design, (EHEDG 2004). The group provides technical support for European legislation that requires that handling, preparation, processing, and packaging of food be done using hygienic machinery and in hygienic premises (CEN 2009 and ISO 2012). EHDG guidelines include accepted materials of construction, instructions for construction of equipment, functional requirements for the prevention of microbial harborage sites and pathogen growth, and instructions for validating the effectiveness of hygienic design of equipment. The organization also performs validation testing on equipment to evaluate cleanability (EHEDG 2004, Schmidt and Erickson 2005, Timmerman 2013). The cleaning validation standards under EHEDG stress finding a balance between theoretically proven methods and practical realization of those methods (Timmerman 2013).
The National Sanitation Foundation (NSF) is involved in developing standards for equipment used in food service and retail worldwide. They have also developed general standards for food processing equipment, and have worked with 3-A SS to develop specific standards for equipment used in meat and poultry processing. NSF also provides a variety of certification, auditing, and training programs (Schmidt and Erickson 2005, Schmidt 2013).

2.2.5 Current industrial mushroom slicing equipment

Most of the slicing equipment within the mushroom industry is manufactured by DutchTecSource, which is headquartered in the Netherlands (DutchTecSource, Beusichem, Netherlands). These mushroom slicers consist of successive disks aligned and separated by spacers. This design is unique in that the slicer blades cut mushrooms parallel to the stem and, according to the manufacturer, minimizes cell damage so that shelf life is improved (DutchTecSource). It is likely that these mushroom slicers would not conform to 3-A SS or EHEDG standards because the many metal-to-metal junctions that occur between blades and spacers would make penetration of cleaning and sanitizing solutions difficult. Additionally, frequent and complete disassembly for cleaning and sanitizing is time consuming and often impractical given the demands for continuous production. A current practice in Canada is to boil the detachable slicer head in water for 1 hour (personal communication Glenn Martin, President of the Canadian Mushroom Growers Association, 2015). Many facilities in the U.S. use similar heating treatments between complete disassembly and thorough cleaning of the slicer. Reports of accelerated slicer corrosion and loss of lubricant oils have been reported. According to Chmielewski and Frank (2013) locations within equipment that are slowest to heat during thermal sanitizing treatment must be investigated when developing heating parameters to include
standard sanitation operating procedures. No studies have been conducted by the mushroom industry or by the slicer manufacturer to determine how effective thermal sanitization is for eliminating *L. monocytogenes* throughout the slicer head. Given customer demands and FSMA mandates that science based control measures must be taken to assure safe processing practices, thermal validation studies are necessary.

### 2.4 Validation of sanitation practices

Validation is defined as “obtaining documented evidence that cleaning and/or disinfection processes are effective at reaching a pre-defined level of hygiene” (Timmerman 2013). The FDA Guidance for Industry: Control of *L. monocytogenes* in Refrigerated or Frozen Ready-to-Eat Foods (FDA 2008) recommends that listericidal control measures be established for foods that support the growth of *Listeria* that consistently destroys viable cells such that the finished food product contains less than 0.04 CFU/g of *L. monocytogenes*. FDA further recommends that listericidal process or product control measures provide an equivalent of at least a 6-log reduction of viable cells of *L. monocytogenes* (FDA 2008) in the final product.

Because of inherent hazards when using human pathogens to conduct validation studies in pilot plants or commercial processing plants, it is advantageous to use a non-pathogenic surrogate microorganism. In the case of *L. monocytogenes* validation studies, *L. innocua* is typically used. However, microbial death kinetics must be compared under conditions similar to that of processing conditions to assure that the surrogate is at least as heat tolerant as the pathogen.

Studies involving milk, meat, and poultry products have used *L. innocua* for thermal resistance studies when the heat resistance of *L. innocua* was previously established as being
equal or greater than that of *L. monocytogenes* (Doyle et al. 2001, Friedly et al. 2008, O’Bryan 2006). Few microbiological validation studies of thermal treatments on food processing equipment have been conducted. The effect of dry and moist heat on deli meat slicers contaminated with *Listeria* have been studied. Crandall et al. (2010) inoculated *L. innocua* (10⁷ CFU/cm²) onto components within the slicing equipment, which was then placed in a dry heat oven at 66 or 80°C for 0.5 to 15 hours. No treatments were successful in achieving a 5-log decrease, but the authors proposed that the process could be used as an overnight kill step.

Lindsay et al. (2013) performed further validation studies on deli meat slicers subjected to a moist hot air treatment at 65°C for 7 hours using a temperature-controlled bread proofer. A pan of water was placed at the bottom of the metal cabinet to increase interior relative humidity. Thermocouples were used to determine the location within the equipment that resulted in the longest time to reach the target temperature. This “cold spot” location was then inoculated with *L. innocua* and subjected to heat treatments. The treatment resulted in decreases in the initial inoculum level (4-5 logs/ml) to below detection limits. The results from these studies indicate that moist heat is more efficient for reducing *Listeria* to safe levels and that less severe temperatures can minimize damage to the equipment.

In its recommendation for controlling *L. monocytogenes* in fruit and vegetable operations, United Fresh Produce Association (UFPA 2013) reported that removable slicer heads can be sanitized by completely immersing the pre-cleaned equipment in hot water. They recommended that the circulating water temperature should be at least 170°C to raise all surfaces within the slicer to at least 160°F for 30 seconds. However, UFPA states “Operations should not just assume that they have the right procedures or that they are being performed correctly. Whatever approach is used, each operation should internally validate its cleaning and sanitizing
procedures by microbial testing”. Furthermore, they caution that equipment should be adequately cleaned prior to moist heat sanitizing to prevent coagulation of proteins which may form a conditioning film which could enhance post treatment biofilm formation (Chmielewski and Frank 2013, UFPA 2013).
CHAPTER 3

Abstract

*Listeria monocytogenes* can colonize processing environments and transfer to food contact equipment. Current industrial mushroom slicers consist of successive cutting disks aligned and separated by spacers. The complex design makes frequent and complete disassembly for cleaning and sanitizing time-consuming and therefore, incompatible with continuous commercial mushroom slicing operations. Current industry practice is to submerge the detached slicer head in hot water at varying temperatures and times. However, this practice has not been validated for elimination of *L. monocytogenes*.

A *L. monocytogenes* cocktail was prepared from selected heat tolerant strains isolated from mushrooms, mushroom production and processing environmental samples, and outbreaks. Planktonic cells heat treated immediately after inoculation were at least as heat tolerant as surface planktonic or surface attached cells incubated for up to 7 days. Thermal death time curves generated for *L. monocytogenes* and *L. innocua* at 50, 60, and 70°C confirmed that planktonic *L. innocua* was at least as heat tolerant as the pathogenic species.

Heat penetration studies were performed by monitoring temperatures at the inner and outer circumference of spacers during submersion of the slicer head in a clean-out-of-place (COP) wash tank filled with water at 55, 65, and 75°C. Similar studies were performed in moist air at 85°C.

A pilot plant validation study was conducted by inoculating the inner spacer circumference with *L. innocua* and subjecting the assembled slicer head in heated water at 55, 65, and 75°C. Heating the inner circumference for 7D minutes demonstrated complete elimination of *L. innocua*, and therefore an expected elimination of *L. monocytogenes*. 
3.1. Introduction

The CDC has estimated that *L. monocytogenes* causes approximately 1,600 illnesses, 1,500 hospitalizations, and 260 deaths annually (USDA 2014). Listeriosis accounts for only 0.02% of all foodborne illness; however, it accounts for 28% of deaths resulting from foodborne illness (Tompkin 2002). The ability of *L. monocytogenes* to survive and grow on food-processing surfaces and equipment allows it to persist for extended periods in the food processing environment (Ryser 2007). Although the application of heat to ready-to-eat produce is not practical from a quality standpoint, thermal disinfection of food contact equipment can be used to effectively control *L. monocytogenes* on (Tompkin et al. 1999, UFPA 2013).

A number of deadly outbreaks attributed to *L. monocytogenes* in fresh produce, including fresh-cut celery, cantaloupes, mung bean sprouts, caramelized apples, and packaged salad have occurred in the past five years (Gaul et al. 2013, UFPA 2013, CDC 2011, CDC 2014, FDA 2015, CDC 2015, FDA 2015, CDC 2016, FDA 2016, Beach 2016). To date, there have been no reported outbreaks of listeriosis associated with the consumption of fresh mushrooms. However, contamination of fresh mushrooms can occur as evidenced by several retail surveys (Gonzalez et al. 2001, Leong et al. 2013, Heisick et al. 1989, Van Netten 1989, Samadpour et al. 2006) and a succession of recalls after *Listeria* was found on fresh sliced mushrooms (FDA 2003, FDA 2006, FDA 2009, CFIA 2008, 2011, 2012, 2014, 2015, 2016).

The presence of *Listeria* spp. in a mushroom packing and slicing facility was recently reported by Murugesan et al. (2015). Of 255 samples taken from non-food contact surfaces over 13 months, 18.8% were positive for *L. monocytogenes*. The pathogen was primarily found on floors and drains in continuously wet washing and slicing areas. Slicing equipment, in particular, is a concern due to the manner in which it was designed. If contaminated and insufficiently
cleaned and sanitized over time, the slicing equipment has the potential to become a reservoir for *L. monocytogenes* that can continuously contaminate mushrooms. Good sanitary design principles are especially important for food contact equipment with surfaces where food residue can drip, drain, diffuse, be drawn, or come in direct contact with nonfood contact surfaces (21 CFR Part 110). Materials used for food contact surfaces should be “smooth, impervious, nontoxic, nonabsorbent, and corrosion resistant under conditions of intended use” (AMI 2014, 3-A 2016, EHEDG 2004, Schmidt 2013). Equipment must also be installed, operated, and maintained properly to ensure effective cleaning and prevent cross contamination (3-A 2016, EHEDG 2004, Schmidt 2013). Equipment must be accessible for inspection, maintenance, cleaning and sanitizing, the procedures of which must be clearly written, designed, and proven effective and efficient (AMI 2014, UFPA 2013).

Most of the slicing equipment within the mushroom industry consists of successive disks aligned and separated by spacers. It is likely that these mushroom slicers would conform to good sanitary design because the many metal-to-metal junctions that occur between blades and spacers would make penetration of cleaning and sanitizing solutions difficult. Additionally, frequent and complete disassembly for cleaning and sanitizing is time consuming and often impractical given the demands for continuous production.

Recently, the United States Congress enacted the Food Safety Modernization Act of 2011 (FSMA) (FSMA 2011), under which FDA wrote the regulation “Current Good Manufacturing Practice and Hazard Analysis and Risk-Based Preventive Controls for Human Food” (21 CFR Part 117). The regulation requires specific new requirements for developing and documenting validated preventive control measures that go far beyond the general requirements established earlier in 21 CFR Part 110. In particular, FSMA mandates science-based validation of preventive
controls, including those for cleaning and sanitizing of food contact equipment (Schmidt 2013). Under this regulation, facilities that slice mushrooms will need to provide evidence that the equipment they use can be effectively cleaned and sanitized so that it will not become a source of contamination.

Tompkin et al. (1999) recommended steam treatments in an enclosed structure for disinfecting difficult to clean food contact equipment. The authors noted that all portions of the equipment should reach at least 160°F (71°C) for an hour or more. For equipment that may be damaged by high temperatures, the authors suggested lower temperature treatments of 145°C (63°C) for longer times. More recently, the United Fresh Produce Association (UFPA 2013) recommended the application of circulating hot water or steam sprays at 180°C for disinfecting food contact surfaces. A general guideline for hard to clean food contact equipment was that the heat treatment should be adequate to raise the temperature of all surfaces to at least 170°C (77°C) or to 160°C (71°C) for at least 30 seconds. However, UFPA states “Operations should not just assume that they have the right procedures or that they are being performed correctly. Whatever approach is used, each operation should internally validate its cleaning and sanitizing procedures by microbial testing”.

The effect of dry and moist heat on deli meat slicers contaminated with *Listeria* have been studied. Crandall et al. (2010) inoculated *L. innocua* (10^7 CFU/cm²) onto components within the slicing equipment, which was then placed in a dry heat oven at 66 or 80°C for 0.5 to 15 hours. No treatments were successful in achieving a 5-log decrease, but the authors proposed that the process could be used as an overnight kill step. Lindsay et al. (2013) later reported at least 4 log/ml reductions in *L. innocua* inoculated onto deli slicers and then treated with moist hot air at 65°C for 7 hours in a temperature-controlled bread proofer. The results from these
studies indicate that moist heat is more efficient for reducing *Listeria* to safe levels and that less severe temperatures can minimize damage to the equipment.

An increasing practice in the mushroom packing industry is to immerse slicer heads in a clean-out-of-place (COP) tank filled with water heated to up to 212°F (100°C). There are reports within the industry that repeated exposure to high water temperatures causes soft metal parts to corrode, seals to become damaged, and bearing lubricants to melt and leak. High temperature treatments also put employees at risk for becoming burned. Also, such treatments have not been scientifically validated as effective for eliminating *L. monocytogenes* from all points in the slicer. Research is therefore needed to study the fate of *L. monocytogenes* during thermal sanitization treatments.

The purpose of this study was therefore to validate the efficacy of thermal sanitization treatments on reducing *L. monocytogenes* on a commercial mushroom slicer. This study consists of three parts: 1) Determine the thermal destruction characteristics of planktonic and surface attached *L. monocytogenes* and identify a *L. innocua* strain as a suitable surrogate organism for pilot plant studies, 2) Investigate the rate at which heat penetrates into the slicer head when subjected to hot water and moist air treatments, and 3) Conduct a food safety pilot plant challenge study using the *L. innocua* surrogate, inoculated onto the mushroom slicer cold spot, to confirm that heat treatments, calculated from laboratory thermal destruction studies, are adequate to achieve adequate pathogen destruction during hot water immersion or heated moist air treatments.
3.2. Materials and Methods

3.2.1 Thermal destruction characteristics of planktonic and surface attached L. monocytogenes and identification of a L. innocua strain as a suitable surrogate organism

3.2.1.1. Selection of strains and preparation of working cultures.

Listeria spp. isolates were sourced from the Penn State Department of Food Science microbial culture collection where they were maintained at -80°C in trypticase soy broth containing 0.6% yeast extract (TSBYE; Difco, Franklin Lakes, NJ) supplemented with 16% glycerol. Fifteen Listeria spp. isolates were chosen for initial heat tolerance screening; 10 L. monocytogenes isolates were obtained from mushrooms or mushroom production environments and 4 were clinical isolates associated with non-mushroom listeriosis outbreaks. A single strain of L. innocua isolated from a mushroom packing and slicing facility was also included (Table 3.1).

Working cultures were grown from stocks by streaking isolates on tryptic soy agar with 0.6% yeast extract (TSAYE; Difco) and incubating at 37°C for 20 hours. An isolated colony was inoculated into 10 ml of TSBYE and then incubated at 37°C for 20 hours and then re-streaked on TSAYE and incubated at 37°C for 20 hours. Working cultures on petri plates were stored for later use at 4°C for up to one month. Fresh 20-hour cultures were grown for each replicated experiment, each yielding approximately $10^9$ CFU/ml.
3.2.1.2. Screening study of *Listeria* spp. strains for heat tolerance and preparation of a multi-strain *L. monocytogenes* cocktail.

For each strain, a single isolated colony was taken from the working culture, inoculated into 10 ml of sterile TSBYE, and incubated at 37°C for 20 hours. On the day of each experiment, glass test tubes filled with 9 ml of single strength (1X) sterile phosphate buffered saline (PBS) were tempered to 60°C in a temperature controlled circulating water bath (Precision™ #260; Thermo Fisher, Waltham, MA). The level of the solution in each of the tubes was kept below the water level in the bath to assure uniform heating.

For each determination, a single tube was removed from the water bath and 1 ml of a 20-hour *Listeria* spp. culture was added. The contents were immediately vortexed for 1 second after which the tube was returned to the water bath. After 6 minutes at 60°C, the tube was removed from the water bath, vortexed, and a 0.1 ml aliquot was pipetted into 0.9 ml PBS before vortexing again. Preliminary studies, determined by inserting a K-type thermocouple (Omega Engineering Inc., Stamford, CT) in the center of an un-inoculated tube, showed that the come-up time after returning the tubes to the water bath was approximately 1 second and that the temperature of the tube contents decreased to 30°C within 1 second of dilution in PBS.

Appropriate serial dilutions were made in PBS before plating in duplicate on TSAYE followed by incubation at 37°C for 24 hours. Comparative heat tolerance for each strain was calculated by subtracting log CFU/ml levels obtained after the heat treatment from cell populations determined from unheated 20-hour cultures. Lower population decreases during the heat treatment corresponded with greater heat tolerance.

The 6 most heat tolerant *L. monocytogenes* strains were used to prepare a microbial cocktail for subsequent experiments. Working cultures of each of the selected strains were used
to prepare individual 20-hour cultures in TSBYE, as described previously. Five ml aliquots of each were combined into a sterile 50-ml plastic centrifuge tube and mixed thoroughly by vortexing.

3.2.1.3. Heat tolerance of planktonic vs. adhered cells L. monocytogenes.

The 6-strain L. monocytogenes cocktail was used in experiments to determine if heat tolerance of surface-adhered or planktonic cells was affected by incubation time. For L. monocytogenes surface-adherence experiments, 1 cm x 1 cm stainless steel coupons were prepared by cutting used stainless steel (#304 grade) mushroom slicer blades (DutchTecSource, Beusichem, Netherlands) with a metal band saw. Used blades that showed signs of scratching, pitting, and staining were used because preliminary studies showed greater adherence of L. monocytogenes compared to new blades. Prior to use, the coupons were soaked overnight in acetone to remove any residual grease, washed in detergent (Alconox, White Plains, NY), and then rinsed with deionized water 5 times (Poimenidou 2009). The coupons were autoclaved at 121°C for 15 minutes prior to use.

Surface-adhered cells were prepared by placing individual sterile stainless steel coupons into glass test tubes each containing 9 ml of sterile TSBYE. One milliliter of the L. monocytogenes cocktail was added and the contents were mixed by vortexing. Each set of tubes was then incubated at 30°C for 0, 1, 2, 5, and 7 days. The incubation temperature of 30°C was selected based on preliminary experiments which showed greater adherence compared to 35°C. After each incubation interval, coupons were removed from tubes with sterile forceps and loosely adhered cells were rinsed by successively dipping them in 3 tubes each containing 10 ml of sterile PBS according to the procedure described by Di Bonaventura et al. (2008). Care was
taken to avoid scraping off surface cells by grasping the edges of the coupon with the forceps. After rinsing, coupons were transferred to new sterile tubes each containing 10 ml of PBS and 12 3 mm sterile borosilicate glass beads (Kimble Chase, Rockwood, TN, USA). The tubes were then heated at 60°C for 6 minutes in a water bath as described above. The contents were then vortexed for 1 minute to dislodge strongly adhered cells based on procedures developed by Oh and Marshall (1995) and Lindsay and von Holy (1997). A preliminary study using un-inoculated tubes showed that the temperature of the tube contents decreased by approximately 8°C during vortexing. Immediately after vortexing, a 0.1 ml- aliquot was taken for serial dilution with PBS and plated on TSAYE as described previously.

For planktonic cells, 1 ml of the L. monocytogenes cocktail was added to 9 ml of TSBYE and incubated at 30°C for 0, 1, 2, 5, and 7 days. After each incubation interval, the planktonic suspension was vortexed and the tubes were heated in a water bath as described previously, except that a sterile stainless steel coupon and 12 sterile 3 mm glass beads were added to the tubes to assure that heating properties would be the same as that for adhered cell treatments. After heating, the tubes were removed from the water bath and vortexed for 1 minute. Planktonic cells were vortexed so that both cell types would be subjected to identical shear effects and therefore possible negative effects on cell viability. Immediate serial dilution with PBS and plating on TSAYE was done as described previously.

3.2.1.4. Listeria spp. thermal destruction kinetic study

One milliliter of the L. monocytogenes cocktail or a 20-hour culture of the L. innocua strain evaluated in the screening study was added to tubes containing 9 ml of PBS that were pre-heated to 50, 60, or 70°C in a heated circulating water bath. At appropriate time intervals, 0.1 ml-
aliquots were removed, serially diluted with PBS, and plated onto TSAYE. Colonies were enumerated after incubating at 37°C for 24 hours. When *L. monocytogenes* or *L. innocua* counts on TSAYE were below the detection limit, samples were enriched in TSBYE by incubating at 37°C for 24 hours and then examined for turbidity. Log mean populations were plotted against heating times and the negative inverse of slopes obtained from the regression lines were used to calculate decimal reduction times (D values) for *L. monocytogenes* and *L. innocua* at each heating temperature. Z values were determined by calculating the negative inverse slope of log D vs. temperature (°C) plot.

3.2.2 Heat penetration study on a commercial mushroom slicer head.

3.2.2.1. Equipment used

A 3-lane dry fresh mushroom slicer (DutchTecSource, Beusichem, Netherlands) was used for this study (Figure 4.1). This equipment is typical of that which is used for commercial slicing of *Agaricus bisporus* mushrooms. The slicer head consisted of a stainless steel (#304) frame assembly (13 cm x 33 cm) which holds into place top and bottom stainless steel (#303) axles each fitted with 21 successive circular stainless steel (#301) blades (18 cm x 0.5 cm) each aligned and separated by stainless steel (#303) spacers (OD 7 cm, ID 4 cm, thickness 7 cm). Alignment of the blades during cutting is maintained by 8 High Modulus Polyethylene (HMPE) slotted plates that are fastened with screws onto the slicer head. The weight of the fully assembled slicer head was 96.7 lbs. (43.9 Kg).

A temperature controlled, insulated, 110 gallon (416.4 liters) capacity clean-out-of-place (COP) wash tank (Pumpmaster Model # PM4; Girton Manufacturing Co., Millville, PA) was
used for this study. Water was recirculated through the tank through a positive displacement pump at approximately 100 gallons (379 Liters) per minute. The flow of water was directed through 2 rows of nozzles located along the bottom, outer length of each side of the tank. The water was heated by passing pressurized steam at 50 pounds per square inch gage (344.7 kilopascals) through stainless steel heat exchanger pipes at the bottom of the tank. The tank was fitted with a stainless steel support rack that was approximately 7 inches (17.8) above the steam heat exchanger pipes.

3.2.2.2. Slicer head heat penetration experiments

All experiments were conducted in the BSL 2 Food Safety Pilot Plant in the Penn State Erickson Food Science Building. Time-temperature data on equipment surfaces and in the wash tank were collected using Type-K thermocouples (Omega Engineering Inc., Stamford, CT) and a datalogger (Model CR 3000 Micrologger, Campbell Scientific, Inc., Logan, UT).

For heat penetration experiments, a set of extra slicer head spacers was obtained from the manufacturer. These spacers were modified by drilling a 0.5 mm hole radially through the outer circumference through to the inner circumference surface (Figure 3.2). The thermocouple was inserted so that the tip was flush against the inner surface so that it would make direct contact with the axle thus allowing adequate clearance between the spacer and the axle for assembly of the slicer head. The space between the inserted thermocouple wire and sides of the drilled hole was sealed with an adhesive sealant (DeltaBOND™, Wakefield Thermal Solutions, Pelham, NH) to secure it and to prevent entry of hot water into the drilled hole. The thermocouple wire on the outer circumference of the spacer was secured to an adjacent blade with a 1¼-inch (3.2-cm) paper binder clip which was used to orient the tip in the correct position. Tip contact to the outer
circumference was verified by visual inspection before and after each heat treatment. Blades and spacers were assembled together by a fastening nut at the end of the axle, which was tightened before each experiment with a digital torque wrench (GEK135, Kyoto Tool Co., Ltd., Kyoto, Japan) to a value of 42 foot-pounds (56.9 Newton-meters).

Three preliminary experiments were conducted prior to the slicer head heat penetration study. In the first experiment, the possibility that heat would conduct through the length of the thermocouple wires immersed in the heated tank water and therefore lead to overestimation of temperature measurements was explored. A 12-inch (30.5 cm) length of the middle section of the thermocouple wire was immersed into 75°C tank water for 5 minutes and the tip of the thermocouple measured ambient air temperature 12 inches (30.5 cm) away from the outer side of the tank. Data were collected for up to 30 minutes.

A second preliminary experiment was conducted to determine potential temperature variations within the COP tank. Four rows of perforated stainless steel panels were placed within and along the length of the tank. Twelve thermocouples were fastened on the panels so that the spatial locations of the tips within the tank were approximately 2, 8, and 14 inches (5.1, 20.3, 35.6 cm) above the heating element, approximately 10 inches (25.4 cm) apart horizontally, and so they were at least 12 inches (30.5 cm) away from the sides of the tank. The thermocouples were placed so that the tips extended approximately 1 inch (2.54 cm) away the panel surfaces. The temperature setting for the COP tank was 65°C. After the panels were placed in the filled COP tank, the tank lid was closed, and the steam was turned on. Data were collected for up to 30 minutes.

A third preliminary experiment was carried out to determine if temperatures on the inner and outer circumference of the spacers were affected during heating by their horizontal location
across the axle. Three thermocouple-fitted spacers were placed along an axle, with one at the mid-point and two others each approximately 5 inches (12.7 cm) toward the ends of each axle. The slicer was reassembled and placed into the COP tank at 65°C and data were collected for up to 30 minutes.

For hot water heat penetration experiments, approximately 100 gallons (379 liters) of water were filled into the tank and heated to 55, 65, or 75°C. The circulating pump was kept on at all times throughout the experiment. The range of water temperatures was selected to include levels that would be lethal to Listeria yet not hot enough to be a hazard to the experimenters. Upon reaching the desired temperature, the slicer head was placed into the tank. In order to ease lifting of the slicer head, metal pipes were inserted into existing holes on the slicer head frame so that two individuals on opposite sides of the slicer head could lift and transfer it into the water without disrupting the placement of the thermocouples. The slicer head was lowered into the preheated tank water and onto the stainless steel supporting rack. Individuals working with the slicer head wore gloves and aprons to ensure safety from sharp blades and hot water. The amount of water in the tank (approximately 100 gallons/379 liters) was based on achieving a water level at least 2 inches (5.1 cm) above the top of the slicer head. After the slicer head was placed into the tank, the lid was immediately closed. Temperature data were collected by the data logger at 5-second intervals as described previously.

For moist air heat penetration experiments, the tank was filled with 40 gallons (151.4 liters) of water so that the water level was above the tank temperature sensor but below the support rack. Placement on the support rack positioned the bottom of the slicer head approximately 1 inch (2.54 cm) above the water level. The water in the tank was heated to 85°C and the tank circulating pump was kept on throughout the experiment. Tank air temperature was
monitored by fastening 4 thermocouples to the previously described stainless steel panels so that their locations were 8 and 14 inches (20.3 and 35.6 cm) above the surface of the hot water. When the air temperature reached 85°C, the lid was opened, the slicer head was transferred into the tank as described previously, and the lid was then closed. Temperature values were collected immediately upon immersion of the slicer head and every 5 seconds thereafter as described previously.

3.2.3. Food safety pilot plant challenge study

The *L. innocua* strain identified previously as a suitable surrogate for *L. monocytogenes* was used for this experiment. Fresh 20-hour cultures, grown as described previously, yielded approximately $10^9$ CFU/ml. The inoculation site chosen for this study was the inner circumference of a spacer on the top axle of the slicer head which was prepared for inoculation using the same cleaning and sterilizing method used for stainless coupons described previously.

Preliminary experiments were conducted to estimate population changes as a result of the drying and attachment period just after inoculation and during assembly, immersion in room temperature water for 2 hours, and disassembly of the slicer head. Spacers were positioned at the ends of the top axle. For enumeration of *L. innocua*, inoculated and treated spacers were swabbed (5 strokes vertically and 5 strokes horizontally) with pre-moistened (PBS) sterile cotton swabs, and the swab tip was dispensed into a test tube containing 10 ml of PBS. The tube was vortexed for 1 minute, and 0.1 ml was serially diluted in PBS, plated onto TSAYE, incubated for 24 hours at 35°C, and then the number of colonies was counted.

For the *L. innocua* challenge study, spacers were disassembled from the slicer head and the inner circumference of duplicate spacers was spot-inoculated with 0.1 ml of the 20-hour *L.*
*l. innocua* culture and allowed to air dry for 2 hours. The slicer head was reassembled as described
previously. Inoculated spacers were placed adjacent to each other along one end of the top axle.
The slicer head was then immersed in the COP tank pre-heated to 55, 65 or 75°C as described
previously. The treatment time began when the inner circumference reached target temperatures
of 50, 60 or 70°C. After the appropriate heating period, the slicer head was immediately
disassembled and the inoculated spacers were removed for microbial analysis. Initial inoculated
*L. innocua* populations were determined by enumerating the 20-hour *L. innocua* prepared culture
used to inoculate spacers, as described previously.

After heating, the slicer head was disassembled and the spacers were removed. This
procedure took approximately 10 minutes during which the spacers had cooled to less than 50°C
and could be easily handled. The cooled spacers were placed in a sterilized glass beaker
containing 225 ml of Modified University of Vermont (UVM; Difco) broth and then incubated
for 30°C for 24 hours. After incubation, 0.1 ml of the UVM broth was transferred to 10 ml of
Fraser Broth (FB; Difco) and incubated for 48 hours. The solution was observed for the
characteristic darkening which indicates the presumptive presence of *Listeria* spp. If no
darkening was observed, the sample was confirmed negative for *L. innocua*. Duplicate positive
controls were prepared by immersing inoculated spacers in UVM and transferring to FB to
ensure that the darkening reaction occurred with the *L. innocua* strain used in this study. A 0.1
ml sample was spread onto Modified Oxford Agar (MOX; Difco), incubated for 24 hours at
35°C, and observed for darkened colonies. If positive, an isolated colony was observed
microscopically to confirm presence of *L. innocua* (USDA FSIS 2013).
3.2.4. Statistical analysis

Each experiment within each study was performed in triplicate. Bacterial populations were converted to log CFU/ml before calculating mean population values. All log values indicate log_{10}. Minitab 17 statistical software (State College, PA) was used to determine significant (\(\alpha = 0.05\)) differences using ANOVA and Tukey’s Honestly Significant Difference (HSD). For the comparison of thermal destruction rates, linear regression analysis was used to determine if the relationship between time and population decrease was significant (\(\alpha = 0.05\)).

3.3. Results and Discussion


Table 3.1 shows serotypes and sources for the L. monocytogenes or L. innocua strains used in the screening study. Relative heat tolerance is measured as population decrease after heating at 60°C for 6 minutes. Comparatively lower log reductions correspond to higher heat tolerance levels. The most heat tolerant L. monocytogenes strains were within serotypes 4b, 1/2a, 1/2b, and 1/2c. L. innocua was significantly (\(P \leq 0.05\)) more heat tolerant than all of the L. monocytogenes strains.

The 6 most heat tolerant L. monocytogenes strains (Table 3.1) were chosen for inclusion in a Listeria cocktail for use in subsequent thermal death kinetics experiments. The serotype 4a isolate was not represented in the cocktail because its heat tolerance was lower than other L. monocytogenes strains. The data showing that the L. innocua isolate was more heat tolerant than
any of the *L. monocytogenes* strains provides initial evidence that it will be suitable as a non-pathogenic surrogate organism in later food safety pilot plant challenge studies.

### 3.3.2. Heat tolerance of planktonic vs. adhered cells *L. monocytogenes*

There is no single sanitation standard operating procedure within the mushroom industry for cleaning and sanitizing slicer heads. Because days or weeks may pass before heads are disassembled for deep cleaning, there is a possibility that *L. monocytogenes* cells may attach and form a biofilm. Therefore, possible changes in heat tolerance of planktonic or surface attached cells was studied as a function of incubation time.

Table 3.2 shows *L. monocytogenes* population levels in un-heated and heated planktonic and stainless steel adhered cells. Day 0 *L. monocytogenes* populations on stainless steel coupons represent cells that were not incubated and therefore only loosely adhered. Significant (*P* ≤ 0.05) population increases occurred for both planktonic and surface adhered cells between 0 and 1 days of incubation. However, no further increases occurred between 1 and 7 days of incubation. Population reductions for each incubation time occurred as a result of heating at 60° C for 6 minutes. However, for both planktonic and stainless steel-adhered cells, heat-induced population decreases after incubating for between 1 and 7 days were either significantly (*P* ≤ 0.05) higher or not significantly (*P* > 0.05) different than for un-incubated (day 0) cells. The results from this study show that un-incubated planktonic and adhered cells have equal or higher heat tolerance compared to cells incubated for up to 7 days. Therefore, fresh 20-hour cultures will provide the most conservative (most heat tolerant) scenario for subsequent thermal degradation kinetic studies.
Moltz and Martin (2004) similarly observed increases in *L. monocytogenes* cell adherence on stainless steel coupons incubated in TSB at 20 or 37°C for between 0 and 1 days with no further increases occurring for up to 4 days of incubation. Norwood and Gilmore (1999) also reported that both planktonic and stainless steel adhered *L. monocytogenes* population levels increased between 0 and 1 day in TSB at 25°C with no further increases between 1 and 2 days. In contrast to the present study, Oh and Marshall (1995) reported that planktonic cells became less heat tolerant and that adhered cells became more heat tolerant with increasing incubation time for up to 7 days. However, in that study (Oh and Marshall, 1995), the nutrient medium was periodically replenished, thus maintaining an optimal supply of nutrients. In the present study, the incubation medium was not replenished in order to simulate conditions on commercial slicing equipment where nutrients from mushroom residues may be at less than optimal levels. Differences in results might also be explained by differences in initial *L. monocytogenes* inoculum level, the growth phase of the cells in the incubation medium, and the incubation temperature.

### 3.3.3. *Listeria* spp. Thermal destruction kinetic study

Table 3.3 shows reductions in *L. monocytogenes* and *L. innocua* populations after heating at 50, 60, or 70°C for up to 150 minutes. Population decreases with increasing time for both *Listeria* species were significant (*P* ≤ 0.05) for all temperatures. Complete inactivation of *L. monocytogenes*, at initial concentrations between 7.28 and 7.92 log CFU/ml, was observed after 90, 15, and 8 minutes at 50, 60, and 70°C, respectively. Complete inactivation of *L. innocua*, at initial concentrations between 8.34 and 8.98 log CFU/ml, was reached after 120, 20, and 8 minutes at the same respective temperatures. Thermal destruction of *L. monocytogenes* and *L.*
*innocua* followed a first order model as evidenced by linear regression coefficient values ($R^2$) of 0.95 or higher (Table 3.3). Therefore decimal reduction values (D values) were calculated to directly compare the heat tolerance (Table 3.4). In general, D and $z$ values of *L. monocytogenes* and *L. innocua* from this study were within the same magnitude as those reported in a review by Doyle et al. (2001) which summarized published data on the heat resistance of *L. monocytogenes* in laboratory culture media. D values at 50, 60, and 70°C for both species increased significantly ($P \leq 0.05$) with each 10°C increase in temperature. D values for thermal destruction of *L. monocytogenes* at 50, 60, and 70°C were significantly lower ($P \leq 0.05$) or not significantly different ($P > 0.05$) than values for *L. innocua* within the same temperature range. The results from this study confirms the earlier conclusion drawn from Table 3.1 that *L. innocua* is a suitable surrogate for *L. monocytogenes* and can be used for later pilot plant challenge studies.

### 3.3.4 Heat penetration study on a commercial mushroom slicer.

Visual inspection of the mushroom slicer head revealed extensive metal to metal junctions where soils and microorganisms could accumulate over time without regular and effective cleaning. Used blades provided by several mushroom slicing facilities showed evidence of visible dried soils where the blade surface comes into contact with the spacers. Of particular concern were junctions between the inner circumferences of spacer and the horizontal axes. These areas may be niche sites where moisture and bacterial cells can enter during operation but may be protected from the short-term action of chemical sanitizers unless the slicer head is completely disassembled. Because they are deep within the structure of the equipment, they are also likely to take the longest time to reach lethal temperatures during thermal sanitization treatments.
The results of the first preliminary experiment showed that the length of the thermocouple wire immersed in hot water did not change tip readings by more than 1°C (data not shown). The second preliminary experiment showed that all locations within the COP tank during heating were within approximately 1°C of each other over the 30-minute interval (data not shown). The third preliminary experiment showed that differences in spacer heating times across the horizontal axle were within approximately 1°C of each other throughout the 30-minute interval (data not shown). From these results, it can be assumed that uniform water temperatures throughout the tank are achieved before the slicer is added, and that a single thermocouple-fitted spacer can be used to monitor temperatures at any point along the top or bottom axle.

The choice of target temperatures was made based on the temperatures used for the thermal destruction study. Water temperatures were 5°C higher than target temperatures in order to ensure a sufficient and continuous driving force for reaching spacer target temperatures in a reasonable amount of time.

For hot water immersion studies, heating profiles for water temperatures of 55, 65, and 75°C for the inner (cold spot) and outer circumference spacers located at the top and bottom axle are shown graphically in Figures 3.4 a, b, and c, respectively. Each figure represents data from the single replicate treatment in which the outer and the inner spacer circumference took the longest time to reach the target temperature. For each water temperature, spacer temperature increases were initially rapid and slowed as the external water temperature was approached. Temperature increases at the outer circumference were more rapid compared to the inner circumference, and spacers on the bottom axle appeared to heat more rapidly compared to those on the top axle. Heat profiles for the moist hot air treatment are shown in Figure 3.5. Compared to the hot water immersion studies, heating at all locations took longer although the same general
heating patterns for the inner and outer circumference and the top and bottom axle were observed.

Table 3.5 shows the actual times required for inner and outer circumference of spacers located at the top and bottom axes to reach the target temperatures of 50, 60, and 70°C. The amount time it took for the temperature of the outer circumference to reach the inner circumference ranged from 0.75 to 1.00 seconds for the top axle and from 0.92 to 1.34 seconds for the bottom axle, which can be attributed to the rate at which heat is conducted through the stainless steel spacer. Confirming the graphical data, the times required to reach target temperatures of 50 and 60°C at both inner and outer spacer locations were significantly higher for the bottom axle compared to the top axle. At 70°C, the same effect was observed, although it was not statistically ($P > 0.05$) significant. Interestingly, when the slicer head was inverted 90°C in 55°C water, the apparent difference in heat penetration rates between the axels occurs was much less pronounced (data not shown). This finding suggests that localized temperature differences on the outer circumference surface occur for spacers at the top and bottom axle. This could have been caused by different water flow profiles at these locations.

In the case of the slicer head heated with moist air at 85°C, times to reach the target temperature of 80°C at both the outer and inner spacer locations (Table 3.6) were significantly ($P \leq 0.05$) higher compared to the water immersion data. Mean times for the outer and inner spacer circumference locations to reach the target temperature did not differ significantly between top and bottom axles ($P > 0.05$). Larger variation between replicates studies for moist air treatments compared to hot water treatments may be due to greater difficulty in maintaining control of the rate of steam injected into the tank.
3.3.5. Food safety pilot plant challenge study

The results of the preliminary study to enumerate recovery of *L. innocua* on the spacers after drying versus after drying, assembly, immersion in water, and disassembly are shown in Table 3.7. The mean reduction of $2.05 \pm 0.10 \log$ CFU/ml from the initial 20-hour inoculation culture was likely due to expected cell death during desiccation, possible penetration of wash water and washing away of cells, and friction induced loses as the spacers were placed on and off the horizontal axle of the slicer head during assembly and disassembly. This value was subtracted from initial inoculum concentration to provide an estimated actual initial concentration of *L. innocua* on the spacers as shown in Table 3.8.

Inoculated spacers were placed on the top axle because this location had been shown to be the slowest to heat (Table 3.6). The spacers were located at the end position of the top axle so that they could be quickly assembled and removed and can be justified because of the preliminary experiment showed that the horizontal location along axles had no effect on heating rates.

The heating times chosen for the inner circumference of the spacers were 93.0, 16.4, and 3.5 minutes at treatment temperatures of 55, 65, and 75°C respectively (Table 3.8). These values were determined by multiplying $D_{50}$, $D_{60}$, and $D_{70}$ values calculated from the thermal destruction study data (Table 3.4) by a factor of 7. This factor was selected because the adjusted initial inoculum levels (Table 3.8) were at least 7.0 log CFU/mL which therefore allowed a measurable determination of at least a 7D reduction.

Results for presence or absence of *L. innocua* in Table 3.8 confirm complete microbial destruction at each treatment temperature, as evidenced by no darkening observed in FB after enrichment in UVM. Therefore, a minimum 7D reduction was achieved for each of the heat
treatments. Because it took approximately 10 minutes to disassemble the heated slicer for microbial analysis of the spacers, it is likely that additional thermal destruction occurred during this period. However this amount of time would be similar to actual conditions in a mushroom facility where the slicer is removed from the COP tank and allowed to cool before returning it to the processing line.

3.4. Conclusions

In this study, *L. monocytogenes* strains collected from mushrooms and mushroom production, packing, and slicing operations were screened for heat tolerance, and the most tolerant were used to prepare a microbial cocktail for use in subsequent experiments. A 20-hour culture of *L. monocytogenes* was found to be at least as heat tolerant as planktonic or surface attached cells incubated for up to 7 days. Therefore, 20-hour planktonic cells were used for calculating decimal reduction times at 50, 60, and 70°C. A strain of *L. innocua* was shown to be the more heat tolerant than 14 individual strains of *L. monocytogenes* when heated at 60°C for 6 minutes and at least as heat tolerant as the 6-strain *L. monocytogenes* cocktail heated at 50, 60, and 70°C for up to 150 minutes. Although heating was not affected by the horizontal position of spacers along the slicer axle, there were differences in heating rates between the top and bottom axles. The pilot plant challenge study demonstrated that at least 7.0-log reductions in *L. innocua*, and therefore *L. monocytogenes*, can be expected at the slicer head cold spot by immersing the equipment in water heated to 55, 65, and 75°C for the times calculated from kinetic studies.
Table 3.1. *Listeria* spp. strains selected for heat tolerance screening, population decreases after heating at 60°C for 6 minutes, and strains selected for inclusion in a 6-strain *L. monocytogenes* cocktail.

<table>
<thead>
<tr>
<th>No.</th>
<th><em>Listeria</em> spp.</th>
<th>Serotype</th>
<th>Source</th>
<th>Population decrease (log CFU/ml)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Selected for cocktail&lt;sup&gt;2&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td><em>L. monocytogenes</em></td>
<td>1/2a</td>
<td>2011 Cantaloupe outbreak, Clinical isolate</td>
<td>4.73 ± 0.29 A B</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td><em>L. monocytogenes</em></td>
<td>1/2a</td>
<td>1989 US Hot Dog Outbreak, Food isolate</td>
<td>4.37 ± 0.32 ABCD</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td><em>L. monocytogenes</em></td>
<td>1/2a</td>
<td>Mushroom slicing facility, Environmental isolate</td>
<td>3.91 ± 0.45 BCD</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td><em>L. monocytogenes</em></td>
<td>1/2a</td>
<td>Fresh whole mushrooms, Food isolate</td>
<td>3.48 ± 0.24 D</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td><em>L. monocytogenes</em></td>
<td>1/2b</td>
<td>Mushroom slicing facility, Environmental isolate</td>
<td>5.08 ± 0.15 A</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td><em>L. monocytogenes</em></td>
<td>1/2c</td>
<td>Sporadic human, Clinical isolate</td>
<td>4.59 ± 0.40 A B</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td><em>L. monocytogenes</em></td>
<td>1/2c</td>
<td>Mushroom slicing facility, Environmental isolate</td>
<td>4.32 ± 0.33 ABCD</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td><em>L. monocytogenes</em></td>
<td>1/2c</td>
<td>Fresh sliced mushrooms, Food isolate</td>
<td>4.29 ± 0.34 ABCD</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td><em>L. monocytogenes</em></td>
<td>1/2c</td>
<td>Fresh sliced mushrooms, Food isolate</td>
<td>4.23 ± 0.16 ABCD</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td><em>L. monocytogenes</em></td>
<td>1/2c</td>
<td>Mushroom slicing facility, Environmental isolate</td>
<td>4.05 ± 0.51 BCD</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td><em>L. monocytogenes</em></td>
<td>1/2c</td>
<td>Mushroom farm, Environmental isolate</td>
<td>4.01 ± 0.09 BCD</td>
<td>-</td>
</tr>
<tr>
<td>12</td>
<td><em>L. monocytogenes</em></td>
<td>4a</td>
<td>Mushroom farm, Environmental isolate</td>
<td>5.00 ± 0.19 A</td>
<td>-</td>
</tr>
<tr>
<td>13</td>
<td><em>L. monocytogenes</em></td>
<td>4b</td>
<td>Mexican soft cheese outbreak, Clinical isolate</td>
<td>4.50 ± 0.07 ABC</td>
<td>-</td>
</tr>
<tr>
<td>14</td>
<td><em>L. monocytogenes</em></td>
<td>4b</td>
<td>Milk outbreak, Scott A, Clinical isolate</td>
<td>3.61 ± 0.30 CD</td>
<td>+</td>
</tr>
<tr>
<td>15</td>
<td><em>L. innocua</em></td>
<td>NA&lt;sup&gt;3&lt;/sup&gt;</td>
<td>Mushroom slicing facility, Environmental isolate</td>
<td>2.52 ± 0.28 E</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>1</sup>Mean population reductions determined from duplicate heat treatments within each of 3 replicate experiments ± standard deviation.

<sup>2</sup>The 5 most heat tolerant *L. monocytogenes* strains were selected for inclusion in the cocktail.

<sup>3</sup>Not applicable.
Table 3.2. Decreases in planktonic and stainless steel adhered *L. monocytogenes* cell populations in samples incubated at 30°C for up to 7 days prior to heating at 60°C for 6 minutes.

<table>
<thead>
<tr>
<th>Incubation time (days)</th>
<th>Planktonic cells (log CFU/ml ± SD)</th>
<th>Adhered cells (log CFU/m ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Un-heated</strong></td>
<td><strong>Heated</strong></td>
</tr>
<tr>
<td>0</td>
<td>6.74 ± 0.22 a²</td>
<td>4.42 ± 0.24</td>
</tr>
<tr>
<td>1</td>
<td>7.20 ± 0.17 b</td>
<td>3.74 ± 0.08</td>
</tr>
<tr>
<td>2</td>
<td>7.23 ± 0.11 b</td>
<td>3.10 ± 0.28</td>
</tr>
<tr>
<td>5</td>
<td>7.29 ± 0.09 b</td>
<td>3.17 ± 0.08</td>
</tr>
<tr>
<td>7</td>
<td>7.13 ± 0.13 b</td>
<td>3.52 ± 0.44</td>
</tr>
</tbody>
</table>

1 Mean population reductions were determined from duplicate heat treatments conducted within each of 3 replicate experiments.
2 Different lower case letters within columns indicate significantly (*P* < 0.05) different population means for un-heated samples.
3 Different upper case letters across rows indicate significantly different means occurring as a result of heat treatments (*P* < 0.05).
Table 3.3. Populations of *L. monocytogenes* and *L. innocua* after treatments at 50, 60, and 70°C.

<table>
<thead>
<tr>
<th>Temp.</th>
<th>Time (min)</th>
<th>Replicate</th>
<th><em>L. monocytogenes</em> (log CFU/ml)</th>
<th><em>L. innocua</em> (log CFU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>50°C</td>
<td>0</td>
<td>7.3</td>
<td>7.62</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>6.62</td>
<td>7.08</td>
<td>7.28</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>6.08</td>
<td>5.99</td>
<td>6.83</td>
</tr>
<tr>
<td>60°C</td>
<td>15</td>
<td>5.65</td>
<td>5.95</td>
<td>6.26</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>5.6</td>
<td>5.08</td>
<td>5.2</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>ND (+)</td>
<td>1.41</td>
<td>1.65</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>ND (-)</td>
<td>ND (-)</td>
<td>ND (-)</td>
</tr>
<tr>
<td></td>
<td>120</td>
<td>ND (-)</td>
<td>ND (-)</td>
<td>ND (-)</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>ND (-)</td>
<td>ND (-)</td>
<td>ND (-)</td>
</tr>
</tbody>
</table>

Slope: -0.09 min⁻¹  
R²: 0.98

| 60°C  | 0          | 7.36      | 7.67                           | 7.83 | 7.62 ± 0.24 | 8.34 | 8.85 | 8.53 | 8.57 ± 0.25 |
| 2     | 5.3        | 6.26      | 6.18                           | 5.91 ± 0.53 | 7.18 | 7.62 | 7.68 | 7.49 ± 0.28 |
| 4     | 4.62       | 5.15      | 5.26                           | 5.01 ± 0.34 | 5.78 | 5.83 | 5.94 | 5.85 ± 0.09 |
| 6     | 3.54       | 3.81      | 3.68                           | 3.68 ± 0.13 | 5. | 5.58 | 5.74 | 5.44 ± 0.39 |
| 8     | 1.95       | 2.92      | 2.08                           | 2.32 ± 0.53 | 4.32 | 4.49 | 4.46 | 4.43 ± 0.09 |
| 10    | ND (-)     | 1.26      | 1.4                            | 0.88 ± 0.77² | 3.71 | 3.62 | 3.85 | 3.73 ± 0.11 |
| 15    | ND (-)     | ND (-)    | ND (-)                         | 0.00 ± 0.00² | 1.48 | 1 | 1.6 | 1.36 ± 0.32 |
| 20    | ND (-)     | ND (-)    | ND (-)                         | 0.00 ± 0.00² | ND (-) | ND (-) | ND (-) | 0.00 ± 0.00² |
| 25    | ND (-)     | ND (-)    | ND (-)                         | 0.00 ± 0.00² | ND (-) | ND (-) | ND (-) | 0.00 ± 0.00² |

Slope: -0.53 min⁻¹  
R²: 0.95

| 70°C  | 0          | 7.28      | 7.92                           | 7.8 | 7.67 ± 0.88 | 8.34 | 8.48 | 8.5 | 8.44 ± 0.09 |
| 2     | 5.8        | 5.66      | 5.34                           | 5.60 ± 0.75 | 4.95 | 5 | 5.62 | 5.19 ± 0.37 |
| 4     | 2.34       | 2.7       | 2.72                           | 2.59 ± 0.41 | 2.91 | 2.88 | 2.4 | 2.73 ± 0.29 |
| 6     | ND (-)     | ND (+)    | ND (+)                         | 0.67 ± 0.58¹² | ND (-) | ND (+) | ND (+) | 0.67 ± 0.58¹² |
| 8     | ND (-)     | ND (-)    | ND (-)                         | 0.00 ± 0.00² | ND (-) | ND (-) | ND (-) | 0.00 ± 0.00² |
| 10    | ND (-)     | ND (-)    | ND (-)                         | 0.00 ± 0.00² | ND (-) | ND (-) | ND (-) | 0.00 ± 0.00² |

Slope: -1.01 min⁻¹  
R²: 0.96

The ND indicates not detectable by plating, with (+) and (-) indicating positive and negative for live culture after enrichment.

¹ Mean population calculated by substituting ND (+) for 1.00 log CFU/ml.
² Mean population calculated by substituting ND (-) for 0.00 CFU/ml.
Table 3.4. D values for *L. monocytogenes* and *L. innocua* determined at 50°, 60°, and 70°C and calculated Z values.

<table>
<thead>
<tr>
<th></th>
<th><em>L. monocytogenes</em></th>
<th><em>L. innocua</em></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD (minutes)</td>
<td>Mean ± SD (minutes)</td>
</tr>
<tr>
<td><strong>D&lt;sub&gt;50&lt;/sub&gt;</strong></td>
<td>11.53 ± 0.36 B&lt;sup&gt;2&lt;/sup&gt;</td>
<td>13.28 ± 0.24 A</td>
</tr>
<tr>
<td><strong>D&lt;sub&gt;60&lt;/sub&gt;</strong></td>
<td>1.90 ± 0.038 C</td>
<td>2.34 ± 0.10 C</td>
</tr>
<tr>
<td><strong>D&lt;sub&gt;70&lt;/sub&gt;</strong></td>
<td>0.99 ± 0.01 D</td>
<td>0.93 ± 0.02 D</td>
</tr>
<tr>
<td><strong>Z value (°C)</strong></td>
<td>18.76</td>
<td>17.33</td>
</tr>
</tbody>
</table>

<sup>1</sup> Mean population reductions were determined from duplicate heat treatments conducted within each of 3 replicate experiments.

<sup>2</sup> Different letters indicate significantly (*P* ≤ 0.05) different population means.
Table 3.5. Time to reach target temperature of outer and in circumference of spacers when immersed in hot water.

<table>
<thead>
<tr>
<th>Target Temperature (°C)</th>
<th>Replication</th>
<th>Time (minutes) to reach target temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Top Axle</td>
</tr>
<tr>
<td></td>
<td></td>
<td>At outer spacer circumference</td>
</tr>
<tr>
<td>50</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.00</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>0.97 ± 0.05 CD</td>
</tr>
<tr>
<td>60</td>
<td>1</td>
<td>1.50</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.33</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1.58</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>1.47 ± 0.13 EF</td>
</tr>
<tr>
<td>70</td>
<td>1</td>
<td>1.75</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1.83</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2.08</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>1.89 ± 0.17 CD</td>
</tr>
</tbody>
</table>

Different letters indicate significantly different means (P<0.05)
Table 3.6. Time to reach target temperature of outer and in circumference of spacers when immersed in heated moist air.

<table>
<thead>
<tr>
<th>Target Temperature (°C)</th>
<th>Replication</th>
<th>Top Axle</th>
<th>Bottom Axle</th>
<th>Difference</th>
<th>Top Axle</th>
<th>Bottom Axle</th>
<th>Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>At outer spacer circumference</td>
<td>At inner spacer circumference</td>
<td>Difference</td>
<td>At outer spacer circumference</td>
<td>At inner spacer circumference</td>
<td>Difference</td>
</tr>
<tr>
<td>80</td>
<td>1</td>
<td>10.8</td>
<td>14.5</td>
<td>3.7</td>
<td>8.8</td>
<td>13.3</td>
<td>4.5</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>19.8</td>
<td>20.6</td>
<td>0.8</td>
<td>18.2</td>
<td>18.2</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>17.8</td>
<td>18.9</td>
<td>1.1</td>
<td>17.2</td>
<td>17.7</td>
<td>0.5</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>16.1 ± 4.7 A</td>
<td>18.0 ± 3.1 A</td>
<td>1.87 ± 1.59</td>
<td>14.7 ± 5.2 A</td>
<td>16.4 ± 2.7 A</td>
<td>1.67 ± 2.47</td>
</tr>
</tbody>
</table>

1 Different letters for outer and inner circumference mean temperatures indicates significantly different means ($P < 0.05$)
Figure 3.3. Spatial orientation of thermocouples for COP tank temperature distribution experiment.
Figure 3.4a. Heat penetration curves for outer and inner circumference locations at spacers positioned on the top or bottom slicer blade axles during immersion of the slicer head in water at 55°C.
Figure 3.4b. Heat penetration curves for outer and inner circumference locations at spacers positioned on the top or bottom slicer blade axles during immersion of the slicer head in water at 65°C.
Figure 3.4c. Heat penetration curves for outer and inner circumference locations at spacers positioned on the top or bottom slicer blade axles during immersion of the slicer head in water at 75°C.
Figure 3.5. Heat penetration curves for outer and inner circumference locations at spacers positioned on the top or bottom slicer blade axles during immersion of the slicer head in moist air at 85°C.
CHAPTER 4. SUMMARY AND RECOMMENDATIONS TO INDUSTRY

4.1 Summary

The first objective in this thesis was to prepare a microbial cocktail consisting of 6 heat tolerant strains of *L. monocytogenes*. The cocktail was used to determine the effect of incubation time on heat tolerance of *L. monocytogenes* in planktonic and surface adhered forms. The results showed that 20-hour (Day 0) planktonic *L. monocytogenes* cells were more heat tolerant than adherent cells incubated in for up to 7 days in nutrient broth. Next, D-values for planktonic *L. monocytogenes* and *L. innocua* in nutrient broth were determined by heating at 50, 60, and 70°C for up to 150 minutes. Results showed that *L. innocua* cells had equal or greater heat tolerance compared to *L. monocytogenes* cells. Therefore, it was concluded that 20-hour planktonic *L. innocua* cells could be used for a subsequent pilot plant slicer head thermal sanitization validation study.

The next objective was to conduct a heat penetration study of a commercial mushroom slicer. The slicer head was subjected to hot water or moist air treatments in a temperature-controlled, recirculating clean-out-of-place (COP) equipment wash tank and temperatures were monitored at the inner and outer circumference of cutting blade spacers using thermocouples and a data logger. It was discovered that spacers on the top axle heated slightly more slowly than those on the bottom axle. Therefore, it was decided that the inner circumference of the spacer on the top axle would be used as the inoculation site in the subsequent pilot plant validation study.

The last objective was to validate temperature and time parameters for destruction of *L. monocytogenes* through a *L. innocua* pilot plant challenge study. The achievable inoculum concentration on the inner circumference and the previously calculated D-values at 50, 60, and 70°C were used to calculate times required to achieve at least 7 log reductions of *L. innocua* at
tank water temperatures of 55, 65, and 75°C. The results of this study showed that complete elimination of *L. innocua* occurred for each of the selected treatment temperatures and times. It was therefore concluded that the same reduction of *L. monocytogenes* can be expected by immersing the mushroom slicer head in water heated to 55, 65, or 75°C for 93.0, 16.4, or 6.5 minutes if the heating time begins once the inner circumference reaches target temperatures of 50, 60, or 70°C, respectively. The study also demonstrated that the same results can be achieved using moist air heated to 85°C and holding for 6.5 minutes beginning when the target temperature 70°C is reached at the spacer inner circumference surface.

### 4.2 Recommendations to industry

These results can be used to make specific recommendations to the mushroom industry on procedures for thermal sanitization of commercial mushroom slicers. Current industry practice for boiling slicer heads for an hour or more are very likely to be effective. However such treatments are far more severe than necessary to eliminate *L. monocytogenes* deep within niche sites. Excessive temperatures no doubt contribute to metal corrosion, damaged seals, and leaking bearing lubricants as has been reported by companies that follow this procedure. Less severe heat treatments, achieved by immersion of slicer heads in hot water or in moist air at the validated times determined in this study can therefore be used to achieve adequate destruction of *L. monocytogenes* while likely increasing the usable life of the slicers. Different validated temperature and time combinations will give processors flexibility for incorporating the thermal sanitization process into their production schedules. For instance, higher temperature and shorter times would allow heat treatments to quickly be conducted, perhaps between production shifts.
Lower temperature and longer time treatments could be done when more time is available and has the added advantage of reducing risks to workers from exposure to very hot water.

The temperature and time parameters validated in this study are conservative in that there are several factors that likely increase the actual total *L. monocytogenes* lethality beyond our calculated estimates. These include:

1. The heat tolerance of the *L. innocua* surrogate was greater than that for *L. monocytogenes* at 50 and 60°C although not significantly different at 70°C.
2. The contribution to total lethality from the increase in temperature of the inner circumference before the target temperature is reached is not accounted for in the recommended times.
3. Since water temperatures were 5°C higher than the target temperature, additional lethality can be expected as inner circumference surface temperatures increase above the target temperature over the course of the entire heat treatment.
4. It is unlikely that *L. monocytogenes* populations on the slicer head would reach levels near 7 logs during production runs, and thus a 7D kill is more than adequate for complete destruction.

Monitoring temperatures at the outer circumference of stainless steel spacers and adding an estimate of the maximum time for the target temperature to reach the inner circumference would eliminate variation in come-up times for wash water tanks of varying volumes, water agitation, and heating capacities. For the spacers used in this study, an additional 3 or 5 minutes for hot water or moist air, respectively, would need to be added to account for the time needed for heat to conduct to the inner circumference of the spacer and the junction with the axle. However, this approach would only be valid only for spacers made of the same metal type and
size, and therefore with the same thermal conductivity characteristics, as the ones used in this study.

Differences in the amount of time for outer circumference locations to reach target temperatures for spacers located at the top and bottom axles lead us to conclude that temperatures should be monitored at the inner circumference of spacers. We recommend that for each slicer sold, the manufacturer fabricate an extra set of spacers. Each spacer in the set should be drilled between the inner and outer spacer surface and fitted with a sealed thermocouple in such a way that the tip would be flush with the inner circumference of the spacer. This approach would take into account differences in wash tank heating characteristics as well as any differences in heat transfer rates that could occur if spacers were fabricated from materials with different thermal conductivity properties, such as different grades of stainless steel or other metals such as brass. In practice, for each slicer and wash tank combination, the operator could begin the 7D process immediately after the inner circumference surface reaches the target temperature. If thermocouples are permanently attached to slicers, the spacers would need to be removed and replaced with regular spacers for production runs. A better alternative would be for each of the temperature monitoring spacers to be fabricated with plug-in sockets so that thermocouples could be inserted each time the slicer head is heat treated and then un-plugged before production begins.

Without regular and frequent cleaning, mushroom debris and organic soil deposits between spacers and blades could accumulate on slicers and possibly lead to biofilm formation. Although we found that cell adherence and incubation time did not affect heat tolerance, possible effects of mature biofilms allowed to form for longer times was not studied. Our recommendations therefore do not preclude periodic disassembly and thorough deep cleaning to
prevent build-up of mushroom debris, scale deposits, and biofilms. It is also recommended that the slicer head be thoroughly cleaned before thermal sanitizing treatments. Many operations add chemical cleaning compounds to wash tank water to facilitate removal of surface adhered mushroom debris. This practice may be combined with the thermal sanitization treatment without adversely affecting microbial reductions. However, operators are advised to consult their chemical supplier to determine if the cleaner is compatible at higher water temperatures with materials used to manufacture the slicer.

There were no specifications from the manufacturer for how tightly the end bolts that hold the spacers and blades on the axle should be fastened. In this study, the bolts were tightened to 42 foot-pounds (56.9 Newton-meters). In practice, the torque on the bolts during regular thermal sanitization should be no higher than that which was used for an initial thermocouple verification study.

Thermal sanitization is only a short term strategy. Further recommendations include design improvements to make disassembly easier so that more frequent deep cleaning can be accomplished without causing unfavorable interruptions to production schedules. Additionally, the number of exposed screw threads and sharp angles should be minimized for easier cleaning. Soft metal used to fabricate bearing covers should be replaced with stainless steel that will not corrode when exposed to high temperatures. The bearing lubricants currently used should be replaced with higher melting point alternative that contains an antimicrobial compound. Furthermore, the bearing seal design should be improved to prevent leakage during heating.

Ideally, the metal-to-metal junctions between blades and spacers, which make cleaning and sanitizing difficult, should be eliminated. A possible solution to this issue may include a departure from removable successive blades and discs. Instead, a single unit consisting of an axle
with appropriately welded blades would prevent potential bacterial niches. A longer-term recommendation is for the mushroom industry to explore the use of alternative slicing technologies that more closely adhere to sanitary design criteria established by governmental or non-governmental regulations and guidelines. For instance, a patented transverse slicing wheel has been proposed for cutting food products (Bucks 2011). This consists of high speed V-shaped belts which properly align and feed the product through a rotating slicing wheel. Stainless steel knives form the spokes of the wheel and provide support to the rim. The cut slices are then released into a discharge chute (Urschel Laboratories Inc., 2013). This design allows for easier disassembly, reduced metal-to-metal junctions, sharp angles, and exposed screw threads. Another alternative is water jet system technologies that pump bacteria free water through narrow orifices at very high pressures in a manner that can slice through food products while eliminating the need for cleaning and sanitizing food contact blades and spacers (KMT Waterjet 2016). Although additional costs would be incurred to purchase such systems, no stoppage in production would be required for replacing or sharpening blades.
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