The Pennsylvania State University
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Department of Food Science

FATE OF LISTERIA MONOCYTOGENES AND SALMONELLA SPP. IN SPHAGNUM
PEAT-BASED CASING SOILS AND PRE-HARVEST FOOD SAFETY
INTERVENTIONS TO REDUCE CONTAMINATION OF FRESH MUSHROOMS

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ABSTRACT

Efforts are continually made in the mushroom industry to reduce the risk of contamination with human pathogens. Numerous random product samplings reveal that *Listeria monocytogenes* and *Salmonella* spp. are found consistently on whole and sliced fresh mushrooms, which has prompted several recalls over the years. The purpose of this study was to investigate multiple hurdles at pre-harvest that might prevent microbial contamination and growth of human pathogens on mushrooms. Two potential hurdles were investigated: natural microbial populations in casing soils and irrigation water supplemented with sanitizers.

Light peat casing soil alone exhibited a suppressive effect on *L. monocytogenes* and *Salmonella* spp., and dark peat casing soils also had a suppressive effect on the pathogens, however, it took approximately 14 days for pathogens to reach the same low levels in the dark peats as they did in the light peat. Indigenous populations of total aerobic bacteria, *Actinomycetes*, and yeasts and molds were significantly lower in dark peat, as compared to the light peat, and with less competition in the dark peats, pathogen levels did not decrease as rapidly as in the light peat.

Combinations of light and dark peat were used in a model mushroom growing system in ratios of 100:0, 80:20, and 60:40 light:dark and inoculated with *L. monocytogenes* and *Salmonella* spp. for a challenge study. Pathogen levels were sampled over the course of the growing cycle and emerging mushrooms were tested for presence of pathogens. Pathogen populations were lowest in the 100:0 light:dark peat and were highest in the 60:40 light:dark peat, however, in all three combinations pathogen levels decreased by at least 3.18 logs between inoculation and harvest. Average frequency of pathogen transfer from soil to mushroom was
between 45 and 66 percent for *Salmonella* spp. and between 53 and 56 percent for *L. monocytogenes*.

To replicate the scenario where mushroom pins are contaminated with *Listeria* and/or *Salmonella* spp., pins in a model mushroom growing system were spot-inoculated with the pathogens and irrigation water was supplemented with chlorine dioxide, sodium hypochlorite, hydrogen peroxide, and Tsunami 100 - a commercial sanitizer containing peroxyacetic acid. When applied to experimentally inoculated mushrooms, log$_{10}$ CFU/g reductions of both pathogens were between 2.5 and 3.9 and there were no significant differences between the control (water) and the sanitizers. Results of this research can be used to provide growers with information about peat casing soils and sanitizers so they can make informed decisions about what practices to use on their farms to grow and sell safe mushrooms.
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CHAPTER 1. INTRODUCTION

1.1. STATEMENT OF THE PROBLEM

Fresh mushrooms are a healthy, popular food that is consumed cooked or raw. Food safety concerns have increased with rising preference for raw consumption. There are steps that can be taken in the pre-harvest stages of mushroom growing that could lower these risks. Commercial growing practices are similar from farm to farm, therefore the results from experimental studies can be used to make recommendations to the entire industry.

1.2. RESEARCH OBJECTIVES

The broad objective of this research is to determine the efficacy of pre-harvest food safety interventions to reduce the risk of contamination of mushrooms with human pathogens. Specific objectives are:

- To compare levels of indigenous microflora found in light peat casing soils and dark peat casing soils.

Cultural enumeration was used to determine if there is a difference in levels of aerobic bacteria, *Actinomycetes*, and yeasts and molds between light and dark peat casing soils. Understanding the indigenous population similarities and differences between these two types of peat may help explain possible differences between survival of human pathogens in these soils.
• To determine the fate of human pathogens inoculated into light and dark peat casing soils while held at commercial conditions.

Microbial populations in the different peat types may affect the fate of human pathogens that could contaminate casing soil during the growing process. By examining the fate of *Listeria monocytogenes* and *Salmonella* spp. inoculated into casing soil, we can compare the ability of dark and light peat to support or suppress the growth of these select pathogens.

• To determine the fate of human pathogens in a model mushroom growing system

Using standard industry practices to grow mushrooms, casing soils will be inoculated with *Listeria monocytogenes* and *Salmonella* spp. and levels will be monitored over time to confirm the effect of peat type on pathogen survival. Using a small scale growing system, the complete growing cycle will be followed. As mushroom fruiting bodies emerge, samples will be taken to determine if pathogen transfer can occur from casing soil to mushrooms.

• To determine the effect of irrigation water supplemented with sanitizers on levels of human pathogens inoculated onto mushrooms.

Pre-harvest application of sanitizers has been studied for effects of shelf life and mushroom quality. The purpose for this research is to determine if sanitizers would be effective in reducing levels of *Listeria monocytogenes* and *Salmonella* spp. and if pathogens can be transferred to the surface of the mushrooms.
CHAPTER 2. LITERATURE REVIEW

2.1. BACKGROUND

2.1.1. Mushroom consumption and economic significance

Commercial mushroom cultivation began in the United States in 1894 in Kennett Square, Pennsylvania and has continued into other areas of Pennsylvania, as well as other parts of the world (Beyer 2003). By the 1930’s, Kennett Square had been aptly named the “Mushroom Capital of the World,” where the area produced nearly 85% of the United States’ mushroom crop. Today, 51% of the nation’s crop is produced in Kennett Square (Historic 2010).

During the 2009-2010 growing season, total sales of the mushroom crop in the United States were 793 million pounds, with an estimated value of $925 million. Specifically, *Agaricus bisporus* sales were valued at $886 million throughout the United States, with the total volume sales reaching an estimated 778 million pounds. Out of this total volume, Pennsylvania accounted for 351 million pounds in the 2009-2010 growing season with an estimated production value of $308 million (NASS 2010).

2.1.2. Growing practices from composting to harvesting

The process of commercially growing mushrooms involves six steps, requiring a total of 14 weeks to complete. These steps are Phase I composting, Phase II pasteurization and conditioning, spawning, casing, pinning, and harvesting.

Phase I begins when materials such as poultry manure, straw-bedded horse manure, soybean meal, hay, and/or wheat straw are combined, mixed, wetted down, and formed into a pile. Phase I composting can either occur on a traditional wharf where rows of compost are turned regularly, or in an aerated bunker system where air is forced up through small holes in a
concrete slab and into the pile. Both of these practices serve to incorporate the necessary step of periodically introducing air into the compost pile. The composting process itself begins when microbes grow and begin fermenting the ingredients in the pile, producing carbon dioxide, ammonia, and heat. The entire Phase I process takes about seven days to complete (Beyer 2003).

Once Phase I is complete, the next step is to pasteurize the compost substrate in order to kill any unwanted microorganisms, insects, pests, and competing fungi. This step takes about one week to complete. Previous research at Penn State University recommends that compost be held for at least 2 hours at 60ºC for an effective pasteurization process (Schisler 1982). Another goal of Phase II pasteurization is to condition the compost by eliminating soluble and gaseous ammonia, which are toxic to mushrooms. During phase II, these compounds are converted to proteins that the mushrooms can readily use for food. The pasteurization process is not a complete sterilization process since it does not kill all microbes present in the substrate. In fact, it specifically supports the survival and growth of thermophilic organisms that remove the ammonia from the system (Royse and Beelman 2007; Beyer 2003).

After phase II is complete, the mushroom substrate is cooled to 75 - 80 ºF before spawn grain is added. Spawn grain consists of pure mycelium cultures inoculated onto sterilized grains and is used to inoculate the substrate for the subsequent spawn run. Colonization of the substrate occurs over a period of 14 to 21 days, producing a web of mycelium throughout the substrate (Beyer 2003).

Sphagnum-based peat moss supplemented with limestone or spent sugar beet lime is the typical formulation for casing soil, although other materials, such as clay loam soil or coir fibers, also may be used (Beyer 2003; Royse and Beelman 2007). The purpose of adding the casing layer on top of the substrate is to trigger the mushrooms to grow as fruiting bodies (Flegg 1956).
A common practice among growers is to incorporate colonized spawn into the casing layer. Known as compost at casing (CAC) or casing inoculum (CI), it’s purpose is similar to spawn grain and is composed of a sterilized mixture of peat, vermiculite and wheat bran that has been colonized by mushroom mycelium (Royse and Beelman 2007). This procedure facilitates growth of the spawn into the casing so that mushrooms will grow more rapidly than they would with no CI in the casing. It also leads to higher mushroom yields during the harvest period and thus, higher yields per year (Beyer 2003).

Irrigation of the crop is considered a delicate operation with no single standard procedure common to all mushroom farms. According to growers, the frequency of irrigation is based on a subjective consideration of moisture content of the substrate, which makes this a highly variable stage in the mushroom growing process. As the mycelium grows through the substrate, networks begin to clump together and mushroom primordia, or pins develop. Primordia may be classified as having a diameter of less than 5 mm with no distinguishable stem and cap. Primordia mature into button mushrooms, which can be classified by a maturity index (Figure 2.1). Mushrooms are typically ready for harvesting 15 to 21 days after casing. Three cycles from pinning to harvesting, known as flushes or breaks, typically occur (Beyer 2003). Once the growing period has ended, growers discard the soil, now called spent mushroom substrate or sell it for other uses.
<table>
<thead>
<tr>
<th>Stage</th>
<th>Approx. diameter of pileus (mm)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;5</td>
<td>“Pinhead”-velum not differentiated</td>
</tr>
<tr>
<td>2</td>
<td>20-30</td>
<td>“Button”-velum visible and intact</td>
</tr>
<tr>
<td>3</td>
<td>30-40</td>
<td>“Closed cup”-velum stretched but still intact</td>
</tr>
<tr>
<td>4</td>
<td>30-40</td>
<td>“Cup”-velum starting to tear</td>
</tr>
<tr>
<td>5</td>
<td>30-50</td>
<td>“Cup”-velum torn, pileus still cup-shaped, gills clearly visible</td>
</tr>
<tr>
<td>6</td>
<td>40-60</td>
<td>“Flat”-upper surface of pileus convex, gill surface flat or slightly concave</td>
</tr>
<tr>
<td>7</td>
<td>50-70</td>
<td>“Flat”-gill surface curving upwards</td>
</tr>
</tbody>
</table>

**Figure 2.1 Classification of development stages of mushrooms by maturity index**
2.2 CONSUMER PREFERENCE TRENDS

Mushrooms are sold fresh or for further processing. In 1980, 46% of mushroom sales in the United States were for processing, while 54% were directed for fresh mushroom sales (NASS 1981). As consumer preferences have changed over the years, fresh and processed mushroom sales have also changed to meet demands. In 2010, only 14% of the mushroom sales were for processing, while 86% were fresh mushroom sales (NASS 2009, 2010). This increasing trend of fresh mushroom consumption reflects general consumer preference for whole, fresh foods over processed and canned foods. In addition to these trends, there has been increasing evidence about the many health benefits of mushrooms, including high antioxidant and vitamin D levels (Beelman 2004).

2.3. CONTAMINATION AND RECALLS DUE TO LISTERIA MONOCYTÓGENES AND SALMONELLA SPP.

In the past, Listeria monocytógenes was primarily known as a contaminant in raw milk, soft cheeses, and ready-to-eat meat products. Concerns about Listeria contamination of raw vegetables had not been studied extensively until 1981, when an outbreak in Canada prompted investigations which determined that cabbage used to prepare coleslaw was the source of the Listeria outbreaks (Beuchat 1996). After this incident, growing concerns about L. monocytógenes contamination of vegetables led to surveys of vegetables to determine incidence of the pathogen on fresh, raw vegetables in supermarkets (Beuchat 1996, Harris et. al 2003, Strapp 2002, Samadpour 1999, Heisick 1989). Results of these surveys indicated that Listeria spp. can be found on many vegetables, including cabbage, cucumbers, potatoes, radishes, lettuce, broccoli, and tomatoes (Harris et. al 2003, Heisick et. al 1989).
The primary source for *Salmonella* spp. is animal feces. Thus, vegetables grown in contaminated soil are at risk for *Salmonella* spp. contamination. Supermarket samples taken of numerous produce items such as celery, cauliflower, carrots, and celery have tested positive for *Salmonella* spp. (Harris et. al 2003; Samadpour 1999).

Pathogen contamination of mushrooms also has been reported. In Minneapolis, Minnesota, 11% of fresh mushroom samples from a supermarket tested positive for *L. innocua* (Heisick et. al 1989). In the Netherlands, 10% of the mushrooms tested in a grocery were found to be contaminated with *L. monocytogenes* (Van Netten 1989). In Seattle, Washington, 1% of retail mushrooms were found to be contaminated with *L. monocytogenes*, and 5% were contaminated with *Salmonella* (Samadpour 1999). In Northern Ireland, *Salmonella* was isolated from mushrooms, casing, and compost (Meikle 2001). There have been several recalls due to periodic testing for these organisms. A brand of sliced mushrooms tested positive for *L. monocytogenes*, resulting in a recall issued by the Georgia Department of Agriculture (FDA 2003). In 2006, a positive test result for *L. monocytogenes* during random product sampling by the Ohio Department of Agriculture prompted a recall of 10,000 cases of fresh sliced mushrooms distributed to seven states (FDA 2006). Two brands of sliced button mushrooms, as well as sliced Italian brown mushrooms, were recalled in 2008 by the Canadian Food Inspection Agency after they were found to be contaminated with *L. monocytogenes* (CFIA 2008). Most recently, in February of 2009, a Pennsylvania mushroom farm recalled packages of Enoki mushrooms that were contaminated with *L. monocytogenes* (Anonymous 2009).

To date, no cases of foodborne illness have been linked to a recall. However, the mushroom industry is concerned about sources of contamination and what steps can be taken to
prevent further occurrences. Concerns are especially high since, as previously stated, most mushrooms are consumed raw or without a cooking step that would eliminate pathogens.

2.4. HUMAN PATHOGENS OF CONCERN IN FRESH MUSHROOMS

2.4.1. *Listeria monocytogenes*

*L. monocytogenes* is a Gram-positive rod-shaped bacterium naturally found in soil and water. Since *Listeria* spp. can be found on continuously wet surfaces, potential reservoirs in food processing environments include drains, rubber conveyor belts, floors, or ceilings (Ryser 2007). Condensate or splashes from the floor or drains of growing rooms could contaminate mushroom surfaces and subsequently, be ingested. *L. monocytogenes* is an organism of concern because each year in the United States, about 1,600 people become infected, and approximately 16% die, as a result of this foodborne infection (CDC 2010).

Symptoms of listeriosis are variable, but typically include fever, muscle aches, nausea, and/or diarrhea. If the disease spreads to the nervous system, convulsions and headaches may occur. Pregnant women infected with *L. monocytogenes* may have a flu-like symptoms and the infection can spread to the baby, resulting in premature delivery or miscarriage. Pregnant women are at an increased risk of listeriosis because their immune systems are weakened. Other individuals with weakened immune systems, such as infants, elderly, and individuals with cancer, AIDS, or other immunocompromising diseases also are at an increased risk for listeriosis (CDC 2010).

Although *L. monocytogenes* infections account for only 0.003% of foodborne illnesses in the United States each year, the severity of the disease is such that 19% of foodborne deaths each
year are due to the pathogen, making it the third leading cause of death due to foodborne illness (Ryser 2007, CDC 2010).

2.4.2. *Salmonella* species

*Salmonella* is a Gram-negative, rod-shaped bacteria found in water and in feces of animals. Transmission to humans is typically foodborne, but also can be through contact with infected animals. The three most common serovars of *Salmonella*, Enteritidis, Typhimurium, and Newport, are reported to cause approximately 50% of *Salmonella* spp. infections each year. Symptoms of infection include diarrhea, fever, and abdominal cramps, and people of all ages are at risk (CDC 2010).

Each year, over 1 million people become infected with *Salmonella* spp., accounting for 11% of all foodborne illness cases in the United States, and 35% of these cases result in hospitalization. It is estimated that approximately 378 deaths in the United States each year are due to *Salmonella* spp. It is the leading cause of death due to foodborne illness, accounting for 28% of total foodborne deaths each year (CDC 2010).

2.5. POTENTIAL SOURCES OF CONTAMINATION THROUGHOUT MUSHROOM PRODUCTION AND KNOWN FOOD SAFETY HURDLES AT EACH PRODUCTION STEP

2.5.1. Phase I composting

In this first stage of mushroom production, the raw materials are held either outside on wharves or in aerated composting bunkers. Because these raw materials include horse manure,
chicken manure, hay, and other organic, nutrient-rich materials, it can be expected that mushroom, insect, nematode, and fungal pests, and perhaps human pathogens, are present (Szmidt 2002). This step of the process cannot be expected to eliminate all pathogens and, as with any process involving raw materials, they should be separated physically from pasteurization substrate to avoid cross-contamination.

2.5.2. Phase II pasteurization

Phase II pasteurization is the step in the process where the raw compost materials are heat-treated to kill any unwanted bacteria and parasites (Royse and Beelman 2007). Since there is no further step to eliminate possible human pathogens, this step could be considered a critical control point in the mushroom production process. Inoculation studies done previously at Penn State University have shown that substrate held at 60°C for 2 hours effectively eliminates populations of *Listeria monocytogenes*, *E. coli* 0157:H7, and *Salmonella* (Weil 2004). This time and temperature combination, performed at the Penn State Mushroom Research Center, can be used as a guideline for commercial mushroom farms.

2.5.3. Casing soil

Casing soil is known to contain high levels of aerobic bacteria, *Actinomycetes*, *Pseudomonas*, yeasts, and molds (Doores et. al 1986). Numerous studies have investigated the microbial aspects of casing soils in relation to mushroom fruitification and have shown that interactions between casing soil microflora and the *Agaricus bisporus* mycelium are complex (Gillman et. al 1994, Cochet et. al 1992, Reddy and Patrick 1990). Because of the high levels of microorganisms, it has been suggested that casing soil be heat-treated to reduce the risk of contamination. However, studies conducted at Penn State have shown that sterilization actually may be conducive to pathogen survival if they were to contaminate the casing soil (Chikthimmah
In this study, when *L. monocytogenes* and *Salmonella* spp. were inoculated into autoclaved and non-autoclaved casing soils, the pathogens survived for an extended period of time in the autoclaved soil, but died off quickly in the non-autoclaved soil (Chikthimmah 2006). It was proposed that indigenous microorganisms normally present in casing soil play an important role in suppressing pathogen growth in the soil.

### 2.5.4. Irrigation

Although *Agaricus bisporus* mushrooms typically grow in an environment with high humidity, they still need to be watered regularly. Municipal or private well water is commonly used for irrigation. The source of irrigation water can have an impact on the safety and quality. Groundwater typically has good microbiological quality, although it should be tested before use. Surface water has variable microbiological quality since it is exposed to runoff from animals and should not be used for irrigation (Steele 2004). The frequency of watering and the amount added to the mushroom beds may vary greatly between farms.

### 2.5.5. Harvest

There are many potential ways that employees can contaminate mushrooms, mostly during the harvesting stage. These ways are related to personal hygiene practices of the employees and neglecting safe food-handling practices such as washing hands and wearing hairnets. Through work done at Penn State University, there have been many programs and training tools developed for employees of mushroom farms, such as the Mushroom General Agricultural Practices, or MGAP program. MGAPs include guidelines to ensure that employees at mushroom farms are given the proper training and background tools to understand the importance of how personal hygiene can have an effect on food safety. MGAPs also include storage of materials, farm sanitation, field packing and protection of harvested mushrooms,
cleaning and sanitizing food contact surfaces, pest control, traceability and product recall, calibration, and other guidelines (MGAP 2011). Through the use of these tools and with proper training, contamination of mushrooms by employees can be prevented.

2.6. SPHAGNUM PEAT-BASED CASING SOILS

2.6.1. Properties of light and dark peat and their use in industry

Sphagnum peat is partially decomposed sphagnum moss found in bogs around the world, notably Canada, Ireland, Germany, and other areas around Europe. These bogs vary in depth, and the type of moss found at different depths is also highly variable. Peat dug from the upper layers of the bog is known as light or blonde peat, while peat dug from the deeper layers (more than 4 feet) is known as dark or black peat (Hoitink et. al 1996). Light peat is less dense and is known to contain higher levels of microorganisms than dark peat (Chen et. al 1988).

While it was common for mushroom growers to use only light peat, anecdotal evidence from industry peat suppliers suggests that approximately 33% of Pennsylvania growers are now using a mixture of light and dark peat for their casing soil. Although the dark peat tends to be more expensive than the light peat, there are several advantages growers have noticed when using some combination of dark and light peat. Mushroom caps appear cleaner when dark peats are used, most likely due to the smaller, more dense particle size characteristic of the dark peats. In contrast, the larger and lighter particles in light peats tend to adhere to the mushroom caps (Noble and Gaze 1995). In addition to the cleaner appearance, dark peat yields a more uniform bed distribution and a more consistent yield throughout the flush cycles. This approach is a major financial advantage for farms since they can harvest mushrooms gradually rather than harvest many at one time within a break period.
2.6.2. *Microbial suppression of casing soils*

Studies have shown that high levels of bacteria are present in peat, and these numbers increase after processing steps to refine the peat moss for commercial use (Cormier et al. 1988). For many years, the occurrence of plant diseases has been studied in relation to the types of soil used to grow the plants. Although peat effects are highly variable, it has been shown that light peats are suppressive to plant pathogens such as *Pythium* spp., *Alternaria*, *Stemphylium*, *Fusarium*, and *Rhizoctonia* (Wolfhechel 1988, Tahvonen 1993). In these studies, Wolfhechel (1988) used light sphagnum peat to evaluate if there was a suppressive effect of the soil on *Pythium* spp. while growing cucumber seeds. When unheated and heat-treated peat soils were inoculated with *Pythium* spp., the disease severity after 7 days was evaluated. A suppressive effect was observed in the untreated casing soil, however, this effect was not as dramatic in soils that had been heat-treated. This observation suggested that the suppressive effect on the pathogen is microbiological. This approach was investigated further by Tahvonen (1993), who also found that light peat was suppressive to the plant pathogens *Rhizoctonia*, *Plenodomus lingam*, and *Alternaria brassicicola*, and that sterilizing the peat rendered it non-suppressive to Fusarium wilt disease.

Dark peat casing soils also have been studied in regard to their suppressiveness of plant pathogens. These studies have shown that the more decomposed dark peat is conducive to survival of plant pathogens. Chen et al. (1988a) looked at the relationship between microbial activity and suppression of the plant pathogen, *Pythium ultimum*. They compared young compost (≤ 4 months) and older (> 4 months) compost mixed with sphagnum peat and perlite to see which was more suppressive to the bacteria in cucumber seedlings. Their findings indicate
that the younger compost was consistently suppressive to the plant pathogen, while the older compost was more conducive to the disease within first three days of the experiment, after which it became suppressive. Levels of indigenous microflora were higher in the younger compost as compared to the older compost, and microbial activity was also higher in the younger compost (Chen et. al. 1988a). The authors concluded that disease severity was negatively correlated with microbial activity, which agrees with other findings where a strong negative correlation was seen between disease severity and microbial activity in peat soils (Chen et. al. 1988b, Inbar et. al. 1991). In peat soils with low microbial activity, there was a higher disease severity, and in soils with high microbial activity, there was a much lower disease severity. This study investigated both light and dark peats, as well as a 1:1 mixture of the two, and their abilities to suppress the plant pathogen *Pythium ultimum*, and found that the highest suppression was seen in the light peat, the lowest in the dark, and intermediate in the mixture of the two. In these assays, available carbon was highest in the light peat and broken down, while decomposed carbon was highest in the dark peat (Inbar et. al. 1991).

Much of the work described could imply that this phenomenon can be translated from plant pathogens to human pathogens, however, little work has been done to actually investigate this phenomenon. Recently, light peat casing soils were evaluated for their effectiveness in suppressing human pathogens introduced into the soil and it was found that light peats are in fact suppressive to human pathogens, as evidenced by 5-log and greater reductions of *Listeria monocytogenes* and *Salmonella* spp. populations (Chikthimmah 2006). Sidhu et. al. (2001) studied the effects of young and old compost on *Salmonella* spp. over a four-day period. They observed that in the younger compost, there was a high inactivation rate of the *Salmonella* spp. (~2 log), whereas in the older, more composted soil, *Salmonella* spp. levels remained constant.
for the first two days and then slowly died off, although levels did not decrease to that observed in the younger compost (Sidhu et al. 2001).

There are several theories as to how and why this suppressive effect is seen in the light peats but not as intensely in the dark peats. In some of these studies, a control soil that had been sterilized and then inoculated was included. The sterilized soil consistently supported the growth of plant pathogens as well as human pathogens thus suggesting that suppressive effects are due to the indigenous microflora present in the soils. Aerobic bacteria, *Actinomycetes*, and fungi were measured in compost over time and levels of aerobic bacteria and *Actinomycetes* gradually declined over time, while fungi levels actually increased by one log unit (Sidhu et al. 2001). Chen et al (1988a), observing similar indigenous microflora results in pathogen suppressive and conducive composts, observed that significantly higher levels of total bacteria, *Actinomycetes*, and fungi were present in the suppressive compost, as compared to the conducive compost during the first two days of the experiment. These higher levels of indigenous microflora appear to be a main factor in suppressiveness of soils on pathogens, and this has been documented in several reviews (Quarles 2001, Hoitink and Fahy 1986, Hoitink et al. 1996).

In addition to the effect of differences in microflora between more composted soils and younger, less composted soils, the role of nutrients also has been studied with respect to microbial suppression. Chen et al (1988 a,b) found that levels of glucose, reducing substances, and total carbohydrates were initially all higher in the conducive peat, but levels dropped in both the conducive and suppressive peat over time. They concluded that lower levels of nutrients present in the suppressive soil was due the higher number of microorganisms which were actively utilizing the nutrients. In the conducive soil, less microbial activity resulted in higher amounts of carbohydrates remaining. Competition between the background microflora and the
introduced pathogen was hypothesized to be the mechanism of suppression of *Pythium* in this study (Chen et. al 1988b).

2.7. SANITIZERS USED IN THE MUSHROOM INDUSTRY

According to the U.S. Food and Drug Administration (FDA)’s Code of Federal Regulations, fresh mushrooms are considered to be “raw agricultural commodities” since they do not receive any type of heating or processing step prior to packaging (21 U.S.C. 321). There are numerous sanitizers that may be applied to raw agricultural commodities as irrigation water supplements; however, some of these sanitizers are regulated with respect to their purposes, preparation methods, application methods, and usage levels. Anecdotal evidence suggests that four specific sanitizers are used most commonly in the mushroom industry: hydrogen peroxide, hypochlorous acid (sodium hypochlorite), chlorine dioxide, and peroxyacetic acid. Post-harvest browning of mushrooms is attributed generally to bacterial spoilage, and studies on wash treatments using sanitizers are aimed at reducing levels of *Pseudomonas* spp. or other spoilage bacteria to improve the shelf life and quality (Doores et. al. 1986, Beelman 1988).

2.7.1. Hydrogen peroxide

According to the FDA, hydrogen peroxide is “Generally Recognized As Safe” (GRAS) when used as a bleaching agent or an antimicrobial agent on foods (21 CRF 184.1366). Depending on the food product, it can be used as a direct food additive at levels ranging from 0.05% to 1.25% (500 to 12,500 ppm) (21 CFR 184.1366). Anecdotal reports from mushroom growers suggest that current usage levels on mushroom farms is between 3% and 5% (30,000 – 50,000 ppm).
Studies have shown that hydrogen peroxide is effective in improving mushroom quality when used as a wash treatment at levels ranging from 30,000 ppm to 50,000 ppm (Sapers et. al. 1999, 2001, Sapers 2001). Chikthimmah et. al. (2005) studied the effect of hydrogen peroxide (350 to 7500 ppm) supplemented in the final irrigation treatment on indigenous microbial populations on fresh mushrooms. Maximum microbial reduction was observed at 7300 ppm, which in turn enhanced post-harvest quality and whiteness of the mushrooms. Treatment with 5000 ppm was less effective and no effect was observed at 350 ppm. No adverse effects on mushroom quality or yield were seen following treatments with the hydrogen peroxide.

Some studies have shown that long exposure times to hydrogen peroxide (3-5%) can cause browning in the mushrooms, and in these studies browning inhibitors were used following hydrogen peroxide treatments (McConnell and Beelman 1991, Sapers et. al. 2001, 1995, Cliffe-Byrnes and O’Beirne 2008). McConnell and Beelman (1991) found that the most effective treatment for reducing microbial counts and controlling browning was 1% hydrogen peroxide combined with 0.1% EDTA as the browning inhibitor. Hydrogen peroxide used at 3% followed by sodium erythorbate-based browning inhibitors also was effective in improving shelf-life and controlling browning (Sapers et. al. 1995, 2001).

2.7.2. Chlorine dioxide

Chlorine dioxide is produced by mixing an aqueous solution of sodium chlorite with any GRAS acid (21 CFR 173.325). It is approved for use as an antimicrobial post-harvest wash for fruits and vegetables, as long as it is used at concentrations at or below 3 ppm and is followed by a potable water rinse or a cooking, canning, or blanching step (21 CFR 173.300). These regulations specify that there must not be any residual chlorine dioxide on the product. On raw
agricultural commodities, acidified sodium chlorite is approved for use as an antimicrobial spray or dip at concentrations of 500 ppm to 1200 ppm, provided treatment is followed by a potable water rinse or a cooking, canning, or blanching step (21 CFR 173.325). Using chlorine dioxide as an antimicrobial, as specified previously, has limitations and requires an Environmental Protection Agency (EPA) registration, however, using chlorine dioxide for other purposes, such as a whitening agent, does not require an EPA registration, although this restriction is only mentioned specifically for use in flour (21 CFR 137.105). These specifications do not list a maximum usage level, only that it “…may be added in a quantity not more than sufficient for bleaching…” or for artificial aging effects. Chlorine dioxide is used in the mushroom industry, although it is thought to be less common than some of the other sanitizers.

Numerous studies have shown that chlorine dioxide is effective against bacteria on fresh vegetables (Guthrie and Beelman 1989, Soloman, et. al. 1991, Beuchat et. al. 2004, Kreske et. al. 2006, Stopforth et. al. 2007). Stopforth and others (2007) tested the efficacy of chlorine dioxide against *L. monocytogenes*, *Salmonella* spp., and *E. coli* O157:H7 inoculated onto leafy greens and found that chlorine dioxide treatments at 1200 ppm resulted in a ~3-log reduction of pathogens, which was greater than reductions using water, hypochlorite solution, acidified hypochlorite solution, and acidic electrolyzed water. When applied in a wash treatment, no adverse effects on the appearance of the leafy greens was observed. A potential disadvantage of using chlorine dioxide on vegetables is that although it has a high initial oxidation potential, contact with organic materials reduces its effectiveness in killing pathogens (Lindsay et. al. 2002, Zhang and Farber 1996, Kreske et. al. 2006).

Chlorine dioxide also has been studied at lower concentrations and was still found to be effective in reducing microbial populations (Kreske et. al. 2006). In this study, apples inoculated
with *Bacillus cereus* were washed with either sterile water, chlorine, chlorine dioxide, or a commercial peroxycacid fruit and vegetable wash (Tsunami 200). Both the chlorine and the chlorine dioxide applied at 100 ppm were effective in reducing populations of *B. cereus*, however, the Tsunami 200 was not effective in reducing populations when applied at the manufacturer’s recommended level of 80 ppm. When the same treatment scheme was conducted on stainless steel coupons, all of the sanitizers were more effective, as compared to the apples. In previous research, Beuchat et. al. (2004) found that contact with organic materials lowers the efficacy of oxidizing sanitizers. Zhang and Farber (1996) showed that chlorine dioxide treatment on lettuce and cabbage resulted in a 0.8-log reduction of *L. monocytogenes* when used at a concentration of 5 ppm, which was a significant reduction compared to the control water treatment used. While chlorine dioxide is measured with respect to the concentration, it is also measured according to its oxidation-reduction potential, or ORP value. Research has shown that chlorine dioxide, or any oxidizing sanitizer, with an ORP value between 650 to 700 mV will kill *E. coli*, *Salmonella* spp., and *L. monocytogenes* in a matter of seconds; ORP values less than 485 can take up to 5 minutes to inactivate these pathogens (Suslow 2004).

Chlorine dioxide has been studied on mushrooms to reduce populations of spoilage bacteria, which subsequently would increase shelf life (Guthrie and Beelman 1989, Beelman 1988, Solomon et. al. 1991, Cliffe-Byrnes and O’Beirne 2008). A commercial chlorine dioxide sanitizer (Oxine) was effective at 200 ppm free chlorine at reducing bacterial populations on mushrooms and increasing shelf life when used as a pre-harvest wash (Beelman 1988). This sanitizer also was effective in a post harvest treatment at 50 ppm in controlling bacterial growth and brown color development due to enzymatic reactions. Solomon and others (1991) found that Oxine (50 ppm) in combination with 0.25% calcium chloride reduced bacterial populations and
degree of browning in mushrooms initially and throughout post-harvest storage. No reduction in production yield was observed. Chlorine dioxide (20 ppm) was more effective than chlorine (52 ppm) at controlling browning due to microbial spoilage of mushrooms (Guthrie and Beelman 1989), however, at 25-50 ppm chlorine dioxide was less effective than 3% hydrogen peroxide at reducing *Pseudomonas* spp. levels on mushrooms in a post-harvest wash (Cliffe-Byrnes and O’Beirne 2008).

### 2.7.3. Peroxyacetic acid

Peroxyacetic acid is an aqueous mixture of acetic acid and hydrogen peroxide, and acts as an oxidizing agent. It is a less common sanitizer but still used on some farms. It is FDA-approved for food contact surfaces and has been shown to kill a wide range of microorganisms. Another desirable property of peroxyacetic acid is that the decomposition products are acetic acid, water, and oxygen, which are all harmless and therefore, would leave no residue. In addition, the concentration needed to be effective is much lower than that of chlorine and other sanitizers (Beyer and Poppiti 2007). Contrasting evidence shows that use of peroxyacetic acid only reduced levels of *L. monocytogenes* on lettuce by <1 log, which was not significant compared to water (Baert et. al. 2009).

Some peroxyacetic acids are used as a commercial solution made specifically for washing fruits and vegetables, such as Tsunami 100 (Ecolab). At the recommended levels, some of these commercial sanitizers are not effective in killing vegetative cells when inoculated onto an organic surface, however, they are effective when used on an inert surface inoculated with pathogens (Kreske et. al. 2006). There are no published studies on the effectiveness of peroxyacetic acid on mushrooms.
2.7.4. Hypochlorous acid

Hypochlorous acid, or sodium hypochlorite, is used as a sanitizer as well as a whitening agent on mushrooms. The compound has been approved as a secondary direct food additive to assist in peeling of fruits and vegetables (21 CFR 173.315). While there are no concentration limitations listed specifically for direct addition to food, general usage levels range from 50 ppm to 200 ppm (Novak et. al. 2003).

Sodium hypochlorite is widely used as a water treatment and studies have shown that it may be effective in reducing levels of bacteria on fresh fruits and vegetables (Zhang and Farber 1996, Beuchat et. al. 2004, Kreske et. al. 2006, Stopforth et. al. 2007). Hypochlorite solutions are typically used at the post-harvest stage for vegetables and fruits by spraying over the product on a continuous belt or within a dump tank. Beuchat et. al. (2004) reported that sodim hypochlorite was effective at 200 ppm for reducing vegetative B. cereus cells by 4.5 log CFU/ml when inoculated onto an organic medium. Kreske and others (2006) found that the sanitizer was effective in reducing levels of B. cereus by 4.52 log CFU on apples when used at concentrations between 50 and 100 ppm. Stopforth and others (2007) reported that chlorine (50 ppm) was effective in reducing populations of L. monocytogenes and Salmonella spp. inoculated onto leafy greens by 2.2 and 2.8 log CFU, respectively. While this reduction was significant, as compared to treatment with water, it was less effective than chlorine dioxide treatments. It should be noted that even when there was a period of time between inoculation and treatment, complete attachment was not observed as evidenced by the observation that sterile water control treatments reduced populations of L. monocytogenes and Salmonella spp. by 1.4 and 1.1 log CFU/gram, respectively. Similarly, in the study by Kreske et. al. (2006), where a period of 22
hours was allowed for attachment prior to treatment, there was still a 1.10-log reduction when washed with deionized water.

Sodium hypochlorite has been studied for its antibacterial effects on mushrooms. When used at 100 ppm and combined with 0.05% calcium chloride and 100 ppm Oxine, the treatment was effective at reducing bacterial populations and brown color development (Beelman 1988). Earlier studies have shown that sodium hypochlorite is effective at 150 ppm in reducing levels of *Pseudomonas tolaasii* inoculated onto mushroom caps (Wong and Preece 1984). However, some studies have shown that sodium hypochlorite may actually induce enzymatic browning (Choi and Sapers 1994, Sapers et al. 2006). Discoloration also has been reported when mushrooms were subjected to 100 ppm hypochlorite (Simon and González-Fandos 2010, Guthrie and Beelman 1989). In a study by Simon and González-Fandos (2010), populations of *Pseudomonas* and bacterial blotch incidence did not significantly decrease after treatments with sodium hypochlorite. Concentrations recommended by Suslow (1997) for treating fresh mushrooms are between 100 and 150 ppm available chlorine, followed by an antioxidant to prevent browning.

### 2.8. PEAT SOIL AND SANITIZER USE AS FOOD SAFETY INTERVENTIONS

According to the Centers for Disease Control (CDC), at least 12 percent of illnesses due to foodborne outbreaks were associated with fresh produce consumption in the 1990’s (FDA 2004). Although many efforts have been made to reduce pathogen contamination of fresh produce items, there are still many incidents of contamination being reported. In the Food and Drug Administration (FDA) Produce Safety Action Plan (FDA 2004), the first objective is to prevent contamination of fresh produce with pathogens. In order to achieve this goal, FDA has
suggested regular review of the “Guide to Minimize Microbial Food Safety Hazards for Fresh Fruits and Vegetables” for updates in research which may provide new technologies or other information to reduce microbial levels. FDA also suggests that companies actively develop commodity specific procedures for safe packing, processing, and preparing products. In addition, guidelines for personal hygiene and special preparation at the level of the retailer or consumer are also suggested as a way to meet this goal of minimizing contamination by prevention. The Hazard Analysis Critical Control Point (HACCP) approach is considered the most effective way to anticipate points in the process where contamination can occur and to implement steps to prevent or eliminate hazards from occurring.

The first step of developing a HACCP plan is to conduct a hazard analysis, where each step of an operation is evaluated to determine any opportunities for pathogens to enter the product or survive and grow in or on the product (FDA 2009). Several potential areas of contamination during mushroom production, such as inadequate pasteurization and cross-contamination, have been addressed with MGAPs, as previously stated. The casing step is the next area to look at in the mushroom growing process, since a heat treatment is not generally applied prior to use and the fate of human pathogens in casing soils during commercial *A. bisporus* growing is not known. The practice of incorporating dark peat into casing soil mixtures may present a new food safety risk if this practice lessens pathogen reduction between casing and harvest and if transfer of pathogens from the casing soil to the mushrooms can occur.

To reduce these risks, a multiple hurdle approach can be used to address both the pathogen levels in soil, as well as the potential for transfer of pathogens to mushrooms. The first step would be to determine the fate of *L. monocytogenes* and *Salmonella* spp. in combinations of light and dark casing soils, and to quantify the reduction of these pathogens between casing and
harvesting. Once established, this approach could be considered the first hurdle. If there is transfer of pathogens to the mushrooms, pathogens may continue to be present on the mushrooms at harvesting and at consumption. To reduce this risk, irrigation water supplemented with sanitizers might decrease or eliminate pathogens on mushrooms, thus constituting a second hurdle.

There have been no studies on reduction of human pathogens in casing soils containing actively growing *A. bisporus* nor on the effects of using dark peat formulations. As previously stated, sanitizers have shown some positive effects on reducing indigenous microorganisms on the mushrooms with corresponding increases in product quality. Therefore in this thesis, the reduction of pathogens with peat-based casing soils, in combination with sanitizer treatments, will be studied to determine if it is possible to reduce the risk of contamination of fresh mushrooms with human pathogens.
CHAPTER 3. INDIGENOUS MICROFLORA IN LIGHT AND DARK PEAT CASING SOILS

3.1. ABSTRACT

Studies were conducted to determine levels of total aerobic bacteria, *Actinomycetes*, and yeasts and molds present in light and dark peat casing soils. Initial levels of indigenous microflora were enumerated in casing soil that had not been inoculated with *A. bisporus*. In casing soil samples inoculated with *A. bisporus*, samples were taken throughout an entire 20-day growth cycle. Both experiments showed that light peat soils contained higher levels of indigenous microflora compared to either of the two dark peat casing soils. Background microflora for different ratios of light and dark peat casing soil formulations inoculated with *A. bisporus* also was enumerated over the course of a growing cycle. Levels of aerobic bacteria, *Actinomycetes, Pseudomonas* spp., and yeasts and molds were all significantly affected by time and peat soil type (*P*<0.05). Aerobic bacteria and *Actinomycetes* levels were highest in the 100% light peat over time, intermediate in the 80:20 light:dark peat soil mixture, and lowest in the 60:40 light:dark peat soil mixture. Statistically significant differences only were observed between 100% light peat soil and the 60:40 light:dark peat soil mixture. Levels of yeasts and molds were similar between all three peat soil mixtures, but levels in the 60:40 light:dark mixture were significantly lower, as compared to the other mixtures. Indigenous microflora naturally present on mushrooms also was evaluated at three different growing stages.

3.2. INTRODUCTION

Light peat is generally used throughout the mushroom industry as the primary ingredient in casing soil. In recent years, growers have begun to incorporate dark peat into soil
formulations because of improvements in mushroom appearance and crop harvest distribution. According to peat suppliers, about one third of mushroom growers are using combinations of light and dark peat in soil formulations despite the higher cost for dark peat. Typical practice in the industry is to use a light:dark peat ratio of 90:10 to 60:40 (Personal communication, Dr. John Pecchia). It is hypothesized that use of dark peat results in overall decreases in levels of background microflora in the mixture, which could render casing soils less microbially suppressive, as compared to pure light peat casing soils. Indigenous microbial populations found on mushrooms are representative of populations found in the casing soil (Doores et. al. 1986, Beelman 1988); therefore the indigenous microflora on mushrooms also was evaluated. The objective of this chapter is to compare levels of indigenous microflora in light and dark peat casing soils separately, as well as in a range of combinations known to be used by growers in the mushroom industry.

3.3. MATERIALS AND METHODS

3.3.1. Mushroom growing materials

Light peat casing soil was obtained from Acadian Peat (New Brunswick, Canada) and the two dark peat casing soils were obtained from Harte Peat (Harte Peat Ltd., Monaghan, Ireland) and Sylvan (Ontario, Canada). The dark peats were formulated by the manufacturers. The Harte dark peat had been supplemented with limestone flour and spent sugar beet lime and the Sylvan peat had been premixed with Canadian lime to raise the pH to approximately 7.0. The Acadian light peat had been amended with calcium carbonate (Pulverized High Calcium Limestone, Con-Lime Inc. Bellefonte, PA, USA) at the Mushroom Test Demonstration Facility (MTDF) according to a standard formulation. Each of the casing soils were mixed with Casing Inoculum
(CI) (Phoenixx, Amycel, Madisonville, TX, USA) in the laboratory. Substrate materials were prepared at the MTDF using standard procedures. Materials were obtained as available by coordinating with the MTDF crop cycle schedules (Crop #’s 3713, 3715, 3809).

3.3.2. Cultural enumeration of light and dark peat casing soils

Enumeration methods of Weil (2004) and Chikthimmah (2006) were used where 100 grams each of Harte dark peat, Sylvan dark peat, or Acadian light peat (referred to as “light peat” hereafter) were combined separately with 900 ml Buffered Peptone Water (BPW, Difco, Sparks, MD, USA) in sterile 7.5 L stainless steel containers and stirred for 2 minutes with a sterile pipette to obtain a $10^{-1}$ dilution. From each container, 11 ml was transferred into a 99 ml BPW dilution blank, and subsequent serial dilutions were made. A 100 µl aliquot was spread plated using sterile glass spreaders onto Plate Count Agar (PCA, Difco, Sparks, MD, USA), Actinomycetes Isolation Agar (AIA, Difco, Sparks, MD, USA), and Dichloran Rose Bengal Chloramphenicol (DRBC, Difco, Sparks, MD, USA) in duplicate and incubated at 35 °C for 48 hours, 25 °C for 72 hours, and 25 °C for 96 hours, respectively. Plates were enumerated for total aerobic bacteria (PCA), Actinomycetes (AIA), and yeasts and molds (DRBC). The experiment was conducted three times.

3.3.3. Cultural enumeration of casing soils in a model mushroom growing system

3.3.3.1. Treatments

Harte dark peat was only used for the dark peat casing soil since indigenous microflora levels between the two dark soils were not significantly different. Three treatment ratios of light:dark peat casing soils were evaluated with light:dark peat ratios of 100:0, 80:20, and 60:40.
The 100:0 light:dark peat represents a traditional light peat soil and the other ratios were chosen to represent intermediate and high usage levels of dark peat.

3.3.3.2. Preparation of the model mushroom growing system

A model mushroom growing system was developed to represent, to the extent possible, a laboratory scale version of growing practices used at the MTDF and therefore, of industry. Several “dry runs” were conducted to determine optimal individual growing containers and growing container parameters (material ratios, placement in chamber, etc.) and to determine efficient sampling methods. A summary of adaptations that were made to grow mushrooms in the laboratory using the MTDF as a model can be found in Appendix A.

Plastic deli containers (32 oz., Fabri-Kal, Greenville, NC, USA) were used to grow mushroom samples. Each container was filled with 350 g tightly packed spawned substrate and 200 g of peat casing soil that had been mixed with 2% CI. Containers were enclosed in a 30-gallon plastic garbage bag (Grip n’ Tie, Wegmans, Rochester, NY, USA) loosely opened at the side to retain humidity and eliminate drafts, and held in a 27 ft³ capacity temperature-controlled chamber (Lunaire, Model # C10632H-6N, TPS Inc., New Columbia, PA, USA). Humidity was maintained using an evaporative humidifier (Herrmidifier Centrifugal Atomizer Humidifier 707U, Model 356686-001C, Sanford, NC, USA) placed at the bottom of the chamber that was run continuously during the entire growing cycle. Humidity and temperature conditions selected were typical of commercial mushroom growing houses; initial values were 99% and 24 °C, respectively. After nine days, the temperature was lowered to 21.5 °C and the plastic garbage bags were removed to allow for increased O₂ and CO₂ diffusion.
The mushroom containers were irrigated in a manner intended to simulate, to the extent possible, standard commercial mushroom growing practices. Casing soils obtained from the MTDF arrived at near saturated moisture levels with initial moisture contents of 78 to 81% (Personal Communication, John Pecchia). Preliminary trials established that the containers had adequate water if the casing soil appeared moist and dark, and if water (~1 ml) came out when a small sample was pinched between the thumb and index finger. If the casing soil appeared dry and light brown, and no water came out when a sample was pinched, then the container would need to be watered. Later in the growing cycle, when the emerging mycelia appeared dry or fuzzy on the soil surface, the containers would be watered (Appendix A). Containers were checked each day for these criteria. This procedure differed somewhat from that conducted at the MTDF where beds are watered every other day. Because the containers used in this study were much smaller than the large growing beds at the MTDF, they dried out more rapidly and often required daily watering to maintain desirable moisture levels. A calibrated spray bottle filled with tap water was used to deliver 8 sprays at each watering, for a total of 7.8 ± 0.4 ml. To eliminate possible location effects on drying rate, container positions were rotated daily within the incubator.

3.3.3.3. Sampling

Casing soil samples were taken throughout the growing cycle and enumerated for levels of total aerobic bacteria, Actinomycetes, Pseudomonas spp., and yeasts and molds. On each sampling day, three containers from each treatment combination were selected randomly. Using a sterile spoon, 25.0 g of casing soil was sampled from each container and transferred into a 400 ml stomacher bag (BagFilter, Interscience, France) in the following manner: To account for
depth, the first 15 grams were obtained from the middle of the container and included the top layer through the bottom, 1 cm above where the substrate was located. The rest of the 25 gram sample was obtained by taking 2.5 gram samples from the surface from four positions: 12 o’clock, 3 o’clock, 6 o’clock, and 9 o’clock around the container. This sampling method was employed to obtain a representative sample of the entire casing layer to avoid inadvertently collecting compost. Each composite 25 g sample per container was stomached for 2 minutes at 200 rpm with 225 ml BPW to make a 10⁻¹ dilution. Subsequent dilutions were made in BPW using 9 ml dilution blanks, and 0.1 ml of each sample was plated onto PCA, AIA, PIA, and DRBC. All samples were plated in duplicate. PCA plates were incubated at 35 °C for 24 h and then enumerated manually. AIA plates and PIA plates were incubated at 30 °C for 48 to 72 hours, and DRBC plates were incubated at 25 °C for 96 hours.

3.3.4. Enumeration of indigenous microflora on mushrooms at three different growth stages

Mushrooms (Table 3.1) were obtained from the MTDF by cutting the pins or mature fruiting bodies from the beds at the base of the stem using a sterile scalpel. Mushrooms within the same size category were combined into one sterile stomacher bag and weighed. BPW was added to obtain a 10⁻¹ dilution. Serial dilutions were made using 1.0 ml transfers, and 0.1 ml of sample was spread-plated using a sterile glass spreader onto Actinomycetes Isolation Agar (AIA) and Pseudomonas Isolation Agar (PIA). Aerobic Plate Count Petrifilm (APC Petrifilm™, 3M, St. Paul, MN, USA) was used to enumerate total aerobic bacteria, and 1.0 ml of sample was added to the Petrifilm for enumeration. All plates and Petrifilm were made in duplicate and incubated at 28 °C for 48 hours and then enumerated manually.
Table 3.1 Classification of mushroom sizes according to maturity index

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<th>Size</th>
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<tbody>
<tr>
<td>Small</td>
<td>5 mm - 12 mm</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Medium</td>
<td>16 mm - 22 mm</td>
<td>1</td>
</tr>
<tr>
<td>Large</td>
<td>28 mm - 39 mm</td>
<td>2</td>
</tr>
</tbody>
</table>

$^a$Maturity index was based on classifications in Figure 2.1.

3.3.5. *Statistical analysis*

For analysis of enumeration data (3.3.2.), results were combined for all three trials and analyzed using a one-way analysis of variance (ANOVA) (SAS 9.1.3, Cary, NC). Using Tukey’s Honestly Significant Difference (Tukey’s HSD), differences between bacterial counts in the types of peat were determined based on a significance level of $P<0.05$. This test also was used to compare microbial levels in mushrooms at different growth stages.

For analysis of enumeration data over time (3.3.3.), an ANOVA was used for serial repeated measures with the mixed procedure and Tukey’s Pairwise Comparison, where effects of subject, time, and subject and time interactions were analyzed (SAS 9.1.3, Cary, NC). Significance for all tests was set at $P<0.05$.

3.4. *RESULTS AND DISCUSSION*

3.4.1. *Levels of aerobic bacteria, yeasts and molds, and Actinomycetes in light and dark peat*

In all three categories of organisms enumerated, counts were highest in the light peat compared to both dark peats (Figure 3.4). Although both types of dark peat had fewer microorganisms than the light peat, differences between the Harte dark peat and the light peat
were significant. There were no significant differences between the two types of dark peat for aerobic bacteria, yeasts and molds, or Actinomycetes. Since there were more significant differences between the Harte dark peat and the light peat, the Harte dark peat was used in Agaricus bisporus model growing system studies described in later chapters. This research confirms previous work done with light peat (Chikthimmah 2006), and shows the microbial differences between light peat and dark peat.

Figure 3.1 Populations of indigenous microflora in peat casing soils.
Error bars represent the standard deviation from the mean from 3 separate trials. Columns with different letters represent significant differences for each microbial category ($P<0.05$).
3.4.2. Levels of indigenous microflora in a model mushroom growing system

Aerobic plate counts (APCs) in all three casing soil mixtures appear to follow a similar pattern, where immediately after the casing soil was mixed and added to the containers, levels were between $10^5$ and $10^6 \log_{10} \text{CFU/gram}$. Levels increased to approximately $10^8 \log_{10} \text{CFU/gram}$ one week after the casing was applied, and levels slightly decreased by day 20, or at time of harvest (Figure 3.2). Between days 7 and 20, the ratio with the most amount of dark peat (60:40 light:dark) had the lowest levels of APC’s compared to the other two treatments, and this was statistically significant (Table 3.2). The 100:0 and 80:20 treatments were not significantly different from each other. Overall, there was a main peat effect on the levels of APCs, which can be seen clearly in the 60:40 light:dark combination compared with the other two, and there was also an overall effect of time on the levels of aerobic bacteria in all three treatment combinations.

Levels of Actinomycetes throughout the course of the growing cycles also were significantly affected by the peat combination, as well as time. Over the course of the growing cycle, Actinomycetes show a similar pattern as the APCs, where levels start at around $10^5$ and $10^6 \log_{10} \text{CFU/gram}$ and then increase to approximately $10^8 \log_{10} \text{CFU/gram}$ after one week. After one week, levels remained fairly constant (Figure 3.3). Although the difference doesn’t appear to be as large between the 100:0 and 60:40 treatments as it did for aerobic bacteria, there was a significant difference between levels of Actinomycetes in these two treatments. Levels of Actinomycetes in the 80:20 light:dark treatment were not significantly different, as compared to the other two treatments.

Yeast and molds, in general, were not present at as high of levels as the other two categories of microflora described (Figure 3.4). There was a slight increase in levels between the
initial counts and day 7 counts, but levels remained fairly constant for all three peat combinations throughout the rest of the crop cycle. There were significant effects of peat type and time on levels of yeasts and molds. Specifically, the levels of the 60:40 light:dark peat were lower than levels in the other two peat combinations. Levels of *Pseudomonas* spp. were significantly higher in the 100:0 light:dark peat casing soil compared to the other two mixtures (Figure 3.5). After Day 3, levels in all three peat types stayed fairly constant through the duration of the growing cycle.

Levels of indigenous microflora in dark peat casing soils are consistently lower when compared to light peat casing soils. Not surprisingly, levels of indigenous microflora in casing soil mixtures containing a relatively high percentage of dark peat are lower, as compared to casing soil mixtures containing a lower percentage of dark peat, or containing no dark peat at all. These findings suggest that the addition of dark peat does lower the background microflora of casing soils.
Figure 3.2 Levels of aerobic bacteria in a model mushroom growing system.
Each data point represents the average of three samples.

Figure 3.3 Levels of Actinomycetes in a model mushroom growing system.
Each data point represents the average of three samples.
Figure 3.4 Levels of yeasts and molds in a model mushroom growing system. Each data point represents the average of three samples.

Figure 3.5 Levels of *Pseudomonas* spp. in a model mushroom growing system. Each data point represents the average of three samples.
Table 3.2 P-values and significant differences between peat combinations

<table>
<thead>
<tr>
<th></th>
<th>Population Effects&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LS-Means comparisons&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day</td>
<td>Peat</td>
</tr>
<tr>
<td><strong>Aerobic bacteria</strong></td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td><strong>Actinomycetes</strong></td>
<td>&lt;0.0001*</td>
<td>0.0032*</td>
</tr>
<tr>
<td><strong>Pseudomonas spp.</strong></td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td><strong>Yeasts &amp; Molds</strong></td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

<sup>a</sup>Effects of changes over time (Day), with respect to treatment (Peat), and interactions between treatment and time (Day*Peat)

<sup>b</sup>Differences of Least Squares Means, \( P < 0.05 \)

*Indicates significant effects or differences within row

### 3.4.3. Indigenous populations on mushrooms

*Actinomycetes* populations in the medium and large mushrooms were significantly lower than in small mushrooms, but there were no other significant differences in populations between mushrooms at the different growth stages (Table 3.3). Levels of aerobic bacteria, *Pseudomonas* spp., and *Actinomycetes* were between \( 10^5 \) and \( 10^6 \) log<sub>10</sub> CFU/gram. Populations of total aerobic bacteria are lower than those found in the casing soils, and slightly lower than levels previously reported, which were between \( 10^6 \) and \( 10^7 \) log<sub>10</sub> CFU/gram (Doore et. al. 1986, Beelman and Guthrie 1989).
Table 3.3 Average microbial populations on mushrooms at different growth stages

<table>
<thead>
<tr>
<th></th>
<th>Small log(CFU/g)</th>
<th>Medium log(CFU/g)</th>
<th>Large log(CFU/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomycetes</td>
<td>6.90 ± 0.03</td>
<td>6.60 ± 0.04</td>
<td>6.45 ± 0.07</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>6.54 ± 0.09</td>
<td>6.46 ± 0.03</td>
<td>6.49 ± 0.08</td>
</tr>
<tr>
<td>Aerobic bacteria</td>
<td>6.13 ± 0.02</td>
<td>5.87 ± 0.04</td>
<td>5.78 ± 0.25</td>
</tr>
</tbody>
</table>

A Means (n=3) with different letters are significantly different within rows (P<0.05).

3.5. CONCLUSIONS

These experiments showed that levels of indigenous microflora are lower in dark peat casing soils at a given time point and over a period of time with multiple measurements. The microflora examined in this chapter may have only been a small portion of the actual number of microflora present in these casing soils, since the methods employed were purely cultural. These experiments also show that over time, levels of indigenous microflora in light and dark peat casing soil combinations are lowest in the combination with the highest amount of dark peat.
CHAPTER 4: FATE OF LISTERIA MONOCYTOGENES AND SALMONELLA SPP.
INOCULATED INTO LIGHT AND DARK PEAT CASING SOILS

4.1. ABSTRACT

A challenge study was performed to determine fate of five strains of *Listeria monocytogenes* and two strains of *Salmonella* spp. in light and dark peat casing soils. Initial inoculation levels for *L. monocytogenes* and *Salmonella* spp. among all peat soil types was ~8.3 log and ~8.1 log\(_{10}\) CFU/gram. Peats were sampled over time and enumerated for both pathogens. Autoclaved peat casing soils were inoculated and sampled over time as well. By day 29, *L. monocytogenes* levels in the non-autoclaved soils were reduced by 5.7, 5.34, and 5.44 logs in the light peat, Harte dark peat, and Sylvan dark peat, respectively. *Salmonella* spp. levels were reduced by 4.72, 3.55, and 3.48 logs in the light peat, Harte dark peat, and Sylvan dark peat, respectively. Pathogen levels in the autoclaved soils did not decline over the course of 29 days. These results show that the light peat casing soil results in the highest log reductions for both *L. monocytogenes* and *Salmonella* spp., however, a suppressive effect is also still observed in both dark peats.

4.2. INTRODUCTION

In the previous chapter, it was determined that populations of indigenous microflora were lower in casing soils that contained dark peat. This observation suggests that the type of peat formulated into casing soil may affect pathogen survival. Therefore, the objective of this study was to determine the fate of human pathogens inoculated into light and dark peat casing soils.
4.3. MATERIALS AND METHODS

4.3.1. Strain selection and culture conditions

*L. monocytogenes* and *Salmonella* spp. were chosen as the two human pathogens to use in the challenge studies in this research because both genera have been isolated from fresh mushrooms. *Salmonella* Enteritidis ATCC 13076 (American Type Culture Collection, Manassas, VA, USA) and *Salmonella* Typhimurium ATCC 14028 were used in this study because these two serovars are the most common in causing human disease (Rounds et. al. 2010). The strains of *L. monocytogenes* used in this study are listed in Table 4.1, along with their origin. Multiple strains of each pathogen were used to account for the possibility that survival and growth property differences may exist between the strains (Scott et. al. 2005).

Each of the seven pathogen isolates were streaked from stock cultures onto Tryptic Soy Agar (TSA, Difco, Sparks, MD, USA) to ensure that there was no contamination and incubated at 35 ± 2 °C for 24 hours. A single colony from each plate was inoculated separately into 50 ml TSB and incubated at 37 °C for 24 hours. Growth curves were constructed previously for each pathogen and coordinated with an optical density reading so that it could be confirmed that the cells were in stationary phase growth at inoculation time (data not shown). Absorbance readings were taken at 625 nm to confirm that the cells were in stationary phase growth, and readings over 1.0 for both *L. monocytogenes* and *Salmonella* spp. after 24 hours of growth were considered in stationary phase, in accordance to preliminary growth curve experiments. Cultures were centrifuged (Avanti J-26 XPI, Beckman Coulter, Brea, CA, USA) for five minutes at 8,000 rpm, resuspended in sterile saline (0.75%, Mallinckrodt, Hazelwood, MO, USA) and combined in a 1:1 ratio into a plastic spray bottle. Preliminary data showed that individual pathogen levels
reached \( \sim 10^9 \) CFU/ml, and inoculation recovery levels were \( \sim 10^8 \) CFU/ml for both *Salmonella* spp. and *L. monocytogenes* in each of the three peats.

### Table 4.1 Strains of *L. monocytogenes* chosen for challenge studies

<table>
<thead>
<tr>
<th>Ribotype or Designation</th>
<th>Serotype(^a)</th>
<th>Lineage(^b)</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>DUP-104-2B</td>
<td>4b</td>
<td>1</td>
<td>Human isolate (obtained from PSU FSL culture collection)</td>
</tr>
<tr>
<td>DUP-1053A(^c)</td>
<td>1/2a</td>
<td>2</td>
<td>Turkey deli meat outbreak (CDC, 2002)</td>
</tr>
<tr>
<td>MTDF-1(^d)</td>
<td>4a</td>
<td>3</td>
<td>Isolated from PSU MTDF Phase I composting area (2010)</td>
</tr>
<tr>
<td>DOD-2009-2(^e)</td>
<td>1/2a</td>
<td>2</td>
<td>Isolated from sliced fresh mushrooms (2002)</td>
</tr>
<tr>
<td>DOD-2009-3(^e)</td>
<td>1/2a</td>
<td>2</td>
<td>Isolated from sliced fresh mushrooms (2002)</td>
</tr>
<tr>
<td>DOD-2009-5(^e)</td>
<td>1/2a</td>
<td>2</td>
<td>Isolated from whole fresh mushrooms (2008)</td>
</tr>
</tbody>
</table>

\(^a\)Confirmed using MVLST (Chen et. al. 2007).
\(^b\)Used only in challenge study described in Chapter 4.
\(^c\)Used in challenge studies described in Chapters 5 and 6.
\(^d\)Obtained from the U.S. Department of Defense Veterinary Food Analysis & Diagnostic Lab, Fort Sam, Huston TX.

### 4.3.2. Preparation and inoculation of casing soils

Casing soils were obtained as described in Chapter 3. A sterile plastic bin was lined with aluminum foil and soil was spread in a single layer on the foil. The pathogen inoculum was sprayed evenly onto the soil under a biological fume hood in the Food Safety Pilot Plant, using the same plastic spray bottle type used for watering. The soil was then mixed with a sterile spoon under the biological fume hood. Five hundred sprays per batch of soil delivered 450 ± 4.5 ml of inoculum which yielded, by calculation, approximately \( 10^9 \) CFU/g.
Adequate soil mixing to ensure uniform distribution of pathogens was verified as follows. After spray inoculation and mixing, three 25-gram soil samples were taken from the upper left corner of the bin, the center of the bin, and from the lower right corner of the bin. These samples were added to 225 ml BPW in a stomacher bag and stomached for 2 min at 200 rpm followed by plating on XLD and MOX agars in duplicate. Plates were incubated for 48 hours at 35 ± 2°C and colonies were enumerated manually. Counts from these plates were not statistically different \((P<0.05)\). Random 25-gram aliquots of the inoculated soil were placed into sterile stomacher bags and the net weight of the sample was determined. The tops of the bags were loosely folded over to maintain humidity while allowing for gas exchange. Incubation was at 22°C to simulate typical mushroom growing temperatures. Typical soil levels measured immediately after inoculation were approximately \(10^8\) CFU/g, indicating that some cell death occurred as a result of the method or that not all cells were recovered.

Samples were taken at initially and after 1, 3, 7, 14, 22, and 29 days. Sampling was more frequent initially because it was expected that the greatest changes in microbial levels would occur on these days. In the laboratory, mushroom fruiting bodies were observed at approximately day 20 of the growing cycle, so the study was extended beyond that to account for variability in growing rate. pH measurements, shown in Table 4.2, were taken initially and after 14 and 29 days using a 1:1 mixture of soil and deionized water (Clesceri et. al. 1998). Casing soil samples were sent to the Penn State Analytical Services Laboratory for analysis of moisture, pH, total carbon, total nitrogen, and other mineral properties (Tables 4.3-4.5) (University Park, PA, USA).
4.3.3. **Conditions and treatments**

Three types of peat casing soils were used in this study; two dark peats and one light peat. The dark peats were obtained from Harte and Sylvan facilities, and the light peat was obtained from Acadian Peat, as described previously (Crop # 3713).

There were three treatments for each of the casing soils; a negative control, a positive control, and the sample treatment. Positive controls were autoclaved for 60 minutes at 121°C in stainless steel containers covered tightly with aluminum foil. Preliminary studies showed that the center of the soils reached the autoclave temperature. After the containers cooled for 20 hours at refrigeration temperatures followed by 4 hours at room temperature, they were inoculated with the pathogen cocktail described previously. The positive controls served to demonstrate that if the pathogens were dying in a casing soil, it was likely because of the indigenous microflora and not some other abiotic factor. Negative controls were prepared in the same manner as the samples, except that they were inoculated with sterile saline (0.75%) with no pathogens. The negative controls were to test if there was any *Salmonella* spp. and/or *L. monocytogenes* present in uninoculated casing soils. Sample treatments contained the *Salmonella* spp. and *L. monocytogenes*.

Samples were prepared as described previously, where each peat soil type was inoculated in bulk before aliquots were added to stomacher bags. All filled stomacher bags were incubated at 22°C to represent average growing temperatures for the 29-day holding period.

4.3.4. **Sampling, enumeration, and enrichments**

On each sampling day, three random stomacher bags were taken from each peat type, as well as one bag for the positive control for each peat type, and one for the negative for each peat.
type. Sterile BPW (225 ml) was added to each bag, and bags were stomached for 2 minutes at 200 rpm each to homogenize the samples so that a liquid aliquot could be removed for dilutions and plating. Preliminary experiments showed that stomaching the samples was more effective than other methods (hand-mixing, using a stirbar, etc.) for recovering the desired pathogens, and that stomaching for 2 minutes yielded the same recovery as that for 5 minutes, 10 minutes, and 20 minutes (data not shown). One ml aliquots were distributed in Lactose and University of Vermont (UVM) pre-enrichment broths for *Salmonella* spp. and *L. monocytogenes*, respectively, and also into 9 ml BPW dilution blanks. For each sample, 0.1 ml was spread plated onto Xylose Lysine Deoxycholate Agar (XLD, Difco; Sparks, MD, USA) and Modified Oxford Agar (MOX, Difco; Sparks, MD, USA) for selective growth of *Salmonella* and *Listeria*, respectively, in duplicate. Plates, as well as primary enrichment broths, were incubated at 35 ± 2 °C; plates were incubated for 48 hours and enrichment broths for 24 hours. Plates that had colonies after this time were counted and pathogens were confirmed using methods described at the end of this section; no further enrichment steps were taken with plates that exhibited colonies. If there was no growth, however, then a loopful of sample from the appropriate Lactose broth tube and from the UVM broth tube was plated onto XLD and MOX, respectively, and plates were incubated at 35 ± 2 °C for 24 hours. In addition, 1 ml of the inoculated Lactose and/or UVM was transferred to the secondary enrichment broths, Selenite Cysteine and Tetrathionate and/or Fraser broth, respectively, incubated at 35 ± 2 °C for 24 hours, and then a loopful was plated onto XLD Agar and/or MOX Agar. These plates were incubated at 35 ± 2 °C for 24 hours. Suspect colonies were taken from two plates per treatment per sampling day. Sampled colonies for both pathogens were confirmed by Gram stain. *Listeria* was further confirmed using a visual immunoassay (VIP Gold for *Listeria monocytogenes*, Biocontrol, Bellevue, WA, USA) and *Salmonella* spp. was
confirmed using a latex agglutination test (FT0203 Salmonella Latex Test Kit, Oxoid, Cambridge, UK). After enrichments, *L. monocytogenes* and *Salmonella* spp. were evaluated qualitatively as either present or absent, while countable plates were enumerated for each peat ratio for each sampling day, and normalized by taking the log$_{10}$ of the counts.

**4.3.5. Statistical analysis**

Bacterial counts from all replications of this experiment were combined. An analysis of variance (ANOVA) was performed for serial repeated measures using the mixed procedure and Tukey’s Pairwise Comparison, where effects of subject, time, and subject and time interactions were analyzed for both pathogens (SAS 9.1.3, Cary, NC). Significance for all tests was set at $P<0.05$. Data was also analyzed using Tukey’s HSD for differences at each time point (SPSS, Chicago, IL).

**4.4. RESULTS AND DISCUSSION**

**4.4.1. Casing soil analysis**

The light peat had the highest carbon:nitrogen ratio, which is expected since it is known that it is less decomposed than dark peat (Table 4.2). Moisture levels and pH between the light peat and both dark peats were similar, however the light peat had the highest percent moisture and the lowest pH compared to the dark peats (Table 4.3, 4.4). Native peat typically has an acidic pH (<5.5) so the near neutral pH values confirm that each of the soils had been supplemented with limestone before use.
Table 4.2  pH measurements of peat casing soils over time

<table>
<thead>
<tr>
<th>Peat</th>
<th>Day</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dark (Harte)</td>
<td>0</td>
<td>6.89 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>7.59 ± 0.02</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>7.51 ± 0.07</td>
</tr>
<tr>
<td>Dark (Sylvan)</td>
<td>0</td>
<td>6.92 ± 0.38</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>7.74 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>7.59 ± 0.16</td>
</tr>
<tr>
<td>Light (Acadian)</td>
<td>0</td>
<td>7.01 ± 0.14</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>7.61 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>7.61 ± 0.06</td>
</tr>
</tbody>
</table>

Each pH measurement represents the average of three readings for each time point.
<table>
<thead>
<tr>
<th>Analyte</th>
<th>Results (As is basis)</th>
<th>Results (Dry wt basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.4</td>
<td>-</td>
</tr>
<tr>
<td>Soluble Salts (1:5 w:w)</td>
<td>0.58 mmhos/cm</td>
<td>-</td>
</tr>
<tr>
<td>Solids</td>
<td>22.8 %</td>
<td>-</td>
</tr>
<tr>
<td>Moisture</td>
<td>77.2 %</td>
<td>-</td>
</tr>
<tr>
<td>Organic Matter</td>
<td>11.2 %</td>
<td>48.9 %</td>
</tr>
<tr>
<td>Total Nitrogen</td>
<td>0.06 %</td>
<td>0.3 %</td>
</tr>
<tr>
<td>Carbon</td>
<td>6.8 %</td>
<td>29.8 %</td>
</tr>
<tr>
<td>C:N Ratio</td>
<td>108.4</td>
<td>108.4</td>
</tr>
<tr>
<td>Ammonium N</td>
<td>9.9 mg/kg</td>
<td>43.4 mg/kg</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Results (As is basis)</th>
<th>Results (Dry wt basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.6</td>
<td>-</td>
</tr>
<tr>
<td>Soluble Salts (1:5 w:w)</td>
<td>1.32 mmhos/cm</td>
<td>-</td>
</tr>
<tr>
<td>Solids</td>
<td>30.3 %</td>
<td>-</td>
</tr>
<tr>
<td>Moisture</td>
<td>69.7 %</td>
<td>-</td>
</tr>
<tr>
<td>Organic Matter</td>
<td>15.7 %</td>
<td>51.8 %</td>
</tr>
<tr>
<td>Total Nitrogen</td>
<td>0.54 %</td>
<td>1.8 %</td>
</tr>
<tr>
<td>Carbon</td>
<td>10.5 %</td>
<td>34.8 %</td>
</tr>
<tr>
<td>C:N Ratio</td>
<td>19.4</td>
<td>19.4</td>
</tr>
<tr>
<td>Ammonium N</td>
<td>1.5 mg/kg</td>
<td>5.0 mg/kg</td>
</tr>
</tbody>
</table>
Table 4.5 Sylvan dark peat soil analysis results

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Results (As is basis)</th>
<th>Results (Dry wt basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.5</td>
<td>-</td>
</tr>
<tr>
<td>Soluble Salts (1:5 w:w)</td>
<td>0.54 mmhos/cm</td>
<td>-</td>
</tr>
<tr>
<td>Solids</td>
<td>24.4 %</td>
<td>-</td>
</tr>
<tr>
<td>Moisture</td>
<td>75.6 %</td>
<td>-</td>
</tr>
<tr>
<td>Organic Matter</td>
<td>11.9 %</td>
<td>48.6 %</td>
</tr>
<tr>
<td>Total Nitrogen</td>
<td>0.18 %</td>
<td>0.7 %</td>
</tr>
<tr>
<td>Carbon</td>
<td>8.1 %</td>
<td>33.0 %</td>
</tr>
<tr>
<td>C:N Ratio</td>
<td>44.7</td>
<td>44.7</td>
</tr>
<tr>
<td>Ammonium N</td>
<td>1.2 mg/kg</td>
<td>5.0 mg/kg</td>
</tr>
</tbody>
</table>

4.4.2. Changes in pathogen levels over time

Throughout the course of this experiment, enrichment steps were only needed in the negative control samples. Levels of *L. monocytogenes* and *Salmonella* spp. in the inoculated samples were within detection limits and thus could be quantified at each sampling period.

As expected, levels of *L. monocytogenes* in the autoclaved soils remained constant between inoculation and the last sampling day (Figure 4.1). This pattern agrees with previous research (Chikthimmah 2006) where pathogens survived for long periods of time in sterilized casing soils, but not in un-heated soils. Comparing populations of *L. monocytogenes* in the autoclaved casing soils, both light and dark, to populations of *L. monocytogenes* in the non-autoclaved casing soils, it is apparent that there is a suppressive effect of the casing soils on the
inoculated pathogen. Since this suppressive effect is only seen in the casing soils that had not been autoclaved, this observation adds evidence to support the hypothesis that indigenous microbial populations explain the suppressive nature of the peat containing casing soils.

Levels of *L. monocytogenes* and *Salmonella* spp. in pathogen-inoculated casing soil that had not been previously autoclaved decreased in all three peat soil types (Figure 4.1 and 4.2). Peat type and time were both significant effects for each pathogen, and both types of dark peat overall had significantly higher levels of *L. monocytogenes* and *Salmonella* spp. over time compared to the light peat (Table 4.6). In both dark peat samples, pathogen levels increased slightly after the first three days. *L. monocytogenes* and *Salmonella* spp. decreased more rapidly in the light peat compared to both dark peats during the first three days of sampling and pathogen levels in dark peat soils increased between days 3 and 7. At days 14 and 29, during which pinning and harvesting would be expected to occur if the system had been colonized by *Agaricus bisporus*, levels of *L. monocytogenes* were not significantly different between light and dark peat soils. At day 14, levels of *Salmonella* spp. were not significantly different between the dark and light peat soil types. By day 29, log reductions for *L. monocytogenes* in the light, Harte dark, and Sylvan dark were 5.17, 5.34, and 5.44 log units, respectively, while *Salmonella* spp. log reductions were 4.72, 3.55, and 3.48, respectively. This observation suggests that there would be no differences in the risk for mushroom contamination from casing soil to occur when dark peat is used in casing formulations.
Figure 4.1 Levels of *L. monocytogenes* inoculated into light and dark peat casing soils.
Data for non-inoculated casing soils is not shown because there were no positive samples. Dark A = Harte dark peat, Dark B = Sylvan dark peat.
Figure 4.2 Levels of *Salmonella* inoculated into light and dark peat casing soils. Data for non-inoculated casing soils is not shown because there were no positive samples. Dark A = Harte dark peat, Dark B = Sylvan dark peat.
Table 4.6 P-values and significant differences between peat soil types

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Population Effects&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LS-Means comparisons&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day vs.</td>
<td>Light vs.</td>
</tr>
<tr>
<td></td>
<td>Peat</td>
<td>Harte</td>
</tr>
<tr>
<td><strong>Listeria monocytogenes</strong></td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td><strong>Salmonella spp.</strong></td>
<td>&lt;0.0001*</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

<sup>a</sup>Effects of changes over time (Day), with respect to treatment (Peat), and interactions between treatment and time (Day*Peat)

<sup>b</sup>Differences of Least Squares Means, $P<0.05$

*Indicates significant effects or differences within row

4.5. CONCLUSIONS

Decreases in human pathogens were observed in both light and dark peat casing soils. Although *L. monocytogenes* and *Salmonella* spp. decreased more rapidly during the first 3 days after inoculation, soil type differences were not significantly different between days 14 and 29. The concern for pathogen transfer from soil to mushrooms could be highest during the time when pins develop which would be approximately at day 14 during the growth cycle.

The comparison between autoclaved and non-autoclaved casing soils strongly suggest that indigenous microorganisms play a role in suppressing foodborne pathogens that might be present in the soil. While there are significant differences in the levels of these pathogens over time with respect to the soil type, it should be noted that the interval between “contamination” and pinning could be crucial. It is possible for mushroom soil contamination to occur at any time. That being said, if this contamination event were to occur, say, 24 hours before pinning, then there may be insufficient time for the $3-5 \log_{10}$ reduction to occur in the soil. Thus, the results from this challenge study represent the scenario where soil contamination with high levels
of pathogens occurred before the application of casing soil and the onset of the mushroom growing cycle.

It is necessary to examine this scenario closer in a model mushroom growing system that completes the life cycle of *Agaricus bisporus* to determine if it plays a role in the fate of pathogens that are introduced into the casing soil. In addition, the casing soil is only of concern because it may be a possible for pathogens to transfer onto the mushrooms from the casing soil. In a model mushroom growing system inoculated with *L. monocytogenes* and *Salmonella*, emerging mushrooms can be tested for these pathogens.
CHAPTER 5: FATE OF LISTERIA AND SALMONELLA IN A MODEL MUSHROOM GROWING SYSTEM

5.1. ABSTRACT

Studies were conducted to determine if the previously observed microbial suppressive effect of peat in casing soils is confirmed in casing soils while Agaricus bisporus is actively growing through a complete life cycle. A model mushroom growing system was therefore developed to more closely simulate commercial growing conditions. Casing soil with light:dark peat in ratios of 100:0, 80:20, and 60:40 were supplemented with A. bisporus casing inoculum and inoculated with L. monocytogenes and Salmonella. Pathogen levels in the casing soils were enumerated between casing and harvesting. Transfer of pathogens from casing soil to mushrooms was determined by testing the mushrooms after harvesting. A significant microbial suppressive effect was observed for all of the soil peat mixtures. Pathogen levels remaining in the casing soil throughout the growing cycle were higher in soils containing greater amounts of dark peat. Direct plating and enumeration of L. monocytogenes and Salmonella on harvested mushrooms indicated that the transfer rate to mushrooms was not significantly affected by peat type with approximately 55% of the mushrooms positive for pathogen contamination.

5.2. INTRODUCTION

Since, in the previous chapter, levels of L. monocytogenes and Salmonella were shown to die most rapidly in the light peat casing soil, it is hypothesized that similar effects may be seen in a model mushroom growing system. It is further hypothesized that pathogens in peat combinations with higher percentages of dark peat will die more slowly compared to those in soils containing lower levels or no dark peat. The objective of this experiment was to determine
the fate of human pathogens in inoculated casing soils using a model mushroom growing system and to determine if pathogen transfer to emerging mushrooms can occur.

5.3. MATERIALS AND METHODS

5.3.1. Strain selection and culture conditions

*L. monocytogenes* and *Salmonella* spp. strains used in this study are the same used in the previous chapter (Table 4.1). The method of maintaining and preparing the cultures for inoculation for this study were as described previously in Chapter 4.

5.3.2. Preparation and inoculation of casing soils

The experiment was replicated three times over approximately a 6 month time period. Casing soil materials were obtained from the MTDF (Crop #’s 3801, 3807, and 3809). Harte dark peat and the Acadian light peat were used for each trial. Batches of neutralized light peat for each trial were obtained from separate shipments. Dark peat batches were obtained from two shipments. Fully spawned *Agaricus* colonized Phase II substrate was obtained from the MTDF. Casing soils were mixed in a sterile plastic bin lined with aluminum foil in the following ratios of light:dark peat (wt/wt): 100:0, 80:20, and 60:40. Each soil combination was spray-inoculated under a biological safety hood in the Food Safety Pilot Plant as described in Chapter 4.

5.3.3. Preparation of containers with colonized substrate

After inoculating the soil in one bin, 2% CI was added to the mixture and Day 0 samples were taken from random areas of the bin as previously described. This was repeated for each
light:dark peat ratio treatment. Containers were filled as previously described in Chapter 3 and inoculated with *Listeria* and *Salmonella*.

### 5.3.4. Conditions and treatments

Containers were arranged and held in the Lunaire and watered as previously described in Chapter 3. The interval between casing and harvesting was 20 days.

### 5.3.5. Soil sampling procedure

On each sampling day, three containers from each treatment were randomly chosen and sampled as previously described in Chapter 3. Samples from the first trial of this experiment were plated only on XLD and MOX to enumerate *Salmonella* and *Listeria*, respectively. In subsequent trials, samples were additionally plated on PIA, AIA, and DRBC.

### 5.3.6. Mushroom sampling procedure

The criterion for sampling mushrooms was that they must have a maturity index between 2 and 6 (Figure 2.1). If upon sampling, a randomly chosen container contained at least one mushroom that met these criteria, all qualifying mushrooms were carefully harvested using a sterile scalpel so that the cut on the stem was made above any adhering casing soil.

### 5.3.7. Enumeration and confirmation of pathogens in soil and mushrooms

Pathogens remaining in casing soils were enumerated as previously described in Chapter 4. The presence of pathogens in mushroom(s) was determined by weighing them into stomacher bags followed by addition of 1:2 dilution (w/w) BPW. Samples were stomached for 2 minutes at
200 rpm and 0.1 ml was spread-plated on XLD and MOX in duplicate. Plates were incubated at 35 ± 2 °C for 48 hours after which colonies were enumerated. Suspect colonies taken from two plates per treatment per sampling day and were confirmed as described in Chapter 4.

5.3.8. Antimicrobial assay for soil and mushroom samples

To test if antimicrobial agents were present in the light peat, dark peat, or mushrooms, and if so, if these agents could contribute to the death of L. monocytogenes and/or Salmonella spp., antimicrobial assays were performed according to previous methods (Siragusa and Cutter 1993, Miller and Cutter 2005, Trinetta et. al. 2010). Overnight cultures of L. monocytogenes and Salmonella spp. were incubated in 10 ml of TSB, separately. After 24 hours, all L. monocytogenes cultures were combined and all Salmonella spp. cultures were combined. One hundred µl of either the Salmonella spp. strains or the L. monocytogenes strains were transferred to 10 ml of semi-soft agar (0.75% agar; Difco) and mixed. Tubes were poured over TSA plates under a biological safety hood and dried for 10 minutes, after which four holes were bored into the surface of the agar using a sterilized corer (3 mm diameter). For casing soil samples, 5 grams of light peat and 5 grams of Harte dark peat were combined with 45 ml BPW and serially diluted. All dilutions were filter-sterilized through a 45 µm filter into sterile tubes, and 20 µl from each dilution was transferred to a separate well on Listeria- and Salmonella-seeded plates, in duplicate. On each plate, the positive control was an aqueous, nisin solution (Nisaplin, 10³ IU/ml; ). All plates were dried under the biological safety hood for 10 minutes and incubated at 35 °C for 24 hours. Plates were examined for zones of inhibition and if there were any, diameters were measured using calipers (Table 6.6). This same procedure was followed for mushroom samples, except instead of using peat, fresh mushrooms of various sizes (see Table
6.2) were filtered through sterile cheesecloth into sterile tubes. Samples were filtered by using pressure from gloved fingers to expel natural water from the mushroom itself through the cheesecloth so that no dilutions were necessary. For small, medium, and large mushrooms, 20 μl of the filtrate was transferred into wells of the Listeria-and Salmonella-seeded plates, in duplicate.

5.3.9. **Statistical analysis**

Bacterial counts from all three trials of this experiment were combined. An analysis of variance (ANOVA) was performed for serial repeated measures using the mixed procedure and Tukey’s Pairwise Comparison, where effects of subject, time, and subject and time interactions were analyzed for both pathogens (SAS 9.1.3, Cary, NC). Significance for all tests was set at $P<0.05$. Data was also analyzed using Tukey’s HSD for differences at each time point (SPSS).

Qualitative data for presence/absence of pathogens on mushrooms in relation to the peat mixture was analyzed using chi-squared test for independence (SAS 9.1.3, Cary, NC) where $P<0.05$.

5.4. **RESULTS AND DISCUSSION**

5.4.1. **Pathogen levels over time**

No pathogens were detected in uninoculated casing soils. Average recovered initial inoculum levels for *L. monocytogenes* and *Salmonella* spp. were approximately $10^7$ log\(_{10}\) CFU for all three trials and for all three peat combinations. There were small but significant ($P<0.05$) differences in initial inoculation levels between the trials (Table 5.1). However, there were no significant differences in inoculation levels within each trial.
Significant decreases in *L. monocytogenes* and *Salmonella* spp. populations occurred between inoculation (day 0) and harvesting (day 20) for all three light:dark peat casing combinations (Figures 5.1 and 5.2, respectively). Pathogen levels throughout the course of the growing period were highest overall in the soil containing the greatest amount of dark peat (60:40), with significant peat effects over time occurring with respect to both *L. monocytogenes* and *Salmonella* spp. (Table 5.2). Log reductions of *L. monocytogenes* in 100:0, 80:20, and 60:40 light:dark peat soil were 3.46, 4.02, and 3.18, respectively. Log reductions of *Salmonella* spp. in 100:0, 80:20, and 60:40 light:dark peat soil were 3.60, 4.36, and 3.54, respectively. For both pathogens, overall mean levels for 100:0 and 60:40 light:dark peat soils were significantly different. *L. monocytogenes* mean levels were also significantly different between 100:0 and 80:20 light:dark peat soils.

*L. monocytogenes* population values for each each peat soil combination were significantly different by day 3. Between days 7 and 14, values for 100:0 light:dark and the 60:20 light:dark peat soils were significantly different. After 20 days, *L. monocytogenes* reduction leveled off and the 100:00 light:dark peat was not significantly different than either dark peat combinations.

Similar results were obtained for *Salmonella* spp. By Day 3, pathogen counts in the 100:0 light:dark peat soil were significantly lower than the 60:40 light:dark peat soil, but were not significantly different than for the 80:20 light:dark soil. Between 7 and 14, there were no significant differences between each peat soil formulation. On Day 20, values for the 100:0 light:dark soil were not significantly different from either soil containing dark peat.

It would be interesting to compare pathogen reductions as affected by the presence of *Agaricus*. However, this comparison cannot be made directly since pathogen decreases in
Agaricus-containing soils (this Chapter) were observed using one type of light peat and one type of dark peat in 3 light:dark peat combinations while without Agaricus (Chapter 4) one type of light peat and two types of dark peat were compared. In addition, the soils studied in this Chapter were watered regularly while in the earlier study, no watering was done. Additionally, the temperature and temperature consistency were different between the two experiments.

Table 5.1 Average initial pathogen recovery levels in each trial

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>Trial 1</th>
<th>Trial 2</th>
<th>Trial 3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean counts(^a) (log CFU/gram)</td>
<td>Mean counts(^a) (log CFU/gram)</td>
<td>Mean counts(^a) (log CFU/gram)</td>
</tr>
<tr>
<td><em>L. monocytogenes</em></td>
<td>7.28 ± 0.12 A</td>
<td>7.07 ± 0.08 B</td>
<td>7.94 ± 0.09 C</td>
</tr>
<tr>
<td><em>Salmonella spp.</em></td>
<td>7.15 ± 0.47 A</td>
<td>7.14 ± 0.05 A</td>
<td>7.57 ± 0.29 B</td>
</tr>
</tbody>
</table>

\(^a\)Means (n=27) with different letters are significantly different within column rows (P<0.05).
Figure 5.1 *L. monocytogenes* levels over time in a model mushroom growing system. Average of three separate trials; error bars indicate standard error.
Figure 5.2 *Salmonella* levels over time in a model mushroom growing system. Average of three separate trials; error bars indicate standard error.
Table 5.2 P-values and significant differences between peat combinations against *L. monocytogenes* and *Salmonella* spp.

<table>
<thead>
<tr>
<th></th>
<th>Population Effects&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LS-Means comparisons&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day</td>
<td>Peat</td>
</tr>
<tr>
<td><strong>Listeria</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Salmonella</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Effects of changes over time (Day), with respect to treatment (Peat), and interactions between treatment and time (Day*Peat)

<sup>b</sup>Differences of Least Squares Means, *P*<0.05

*Indicates significant effects or differences within row

---

### 5.4.2. Indigenous microflora of light and dark peat casing soils inoculated with pathogens

Overall, *Pseudomonas* spp., *Actinomycetes*, and molds and yeasts change little during the course of the growing cycle levels, with overall mean populations between days 0 and 20 of 6.79 ± 0.18, 7.74 ± 0.21, and 5.38 ± 0.27 log<sub>10</sub> CFU/gram, respectively (Figures 5.3 – 5.5). Peat type and time did not have significant overall effects on indigenous microflora in inoculated soils (Table 5.3). Overall, pH values dropped slightly between days 1 and 7 but were not significantly affected by peat type. This observation may explain why pathogen reductions were not markedly different in the different peat soil combinations.
Figure 5.3 Indigenous *Pseudomonas* spp. levels over time in a model mushroom growing system with *L. monocytogenes* and *Salmonella* spp. inoculated into soil. Results shown represent the average of two separate trials.
Figure 5.4 Indigenous Actinomycetes levels over time in a model mushroom growing system with *L. monocytogenes* and *Salmonella* spp. inoculated into soil. Results shown represent the average of two separate trials.
Figure 5.5 Indigenous yeast and mold levels over time in a model mushroom growing system with *L. monocytogenes* and *Salmonella* spp. inoculated into soil. Results shown represent the average of two separate trials.
Figure 5.6 pH measurements over time in a model mushroom growing system with *L. monocytogenes* and *Salmonella* spp. inoculated into soil. Results shown represent the average of two separate trials.
Table 5.3 P-values and significant differences between indigenous microflora populations

<table>
<thead>
<tr>
<th>Population Effects</th>
<th>LS-Means Comparisons</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day</td>
</tr>
<tr>
<td>Actinomycetes</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Pseudomonas spp.</td>
<td>0.0003*</td>
</tr>
<tr>
<td>Yeasts &amp; Molds</td>
<td>&lt;0.0001*</td>
</tr>
</tbody>
</table>

*Effects of changes over time (Day), with respect to treatment (Peat), and interactions between treatment and time (Day*Peat)

bDifferences of Least Squares Means, P<0.05

*Indicates significant effects or differences within row

5.4.3. Transfer of pathogens to mushrooms

Despite the fact that \emph{L. monocytogenes} and \emph{Salmonella} spp. populations decreased between inoculation (day 0) and harvesting (day 20), 45-66% of the mushroom samples were positive for pathogen transfer (Tables 5.4a and 5.4b). The average frequency of \emph{L. monocytogenes} and \emph{Salmonella} spp. transfer in the 100:0 light:dark peat mixture was 56% and 66%, respectively. These results are higher than the average transfer frequency seen in the 60:40 light:dark peat, which was 53% for both pathogens. Although these frequencies vary slightly between treatments, this variance is not significant. Therefore, the frequency of mushroom containers positive for \emph{L. monocytogenes} and/or \emph{Salmonella} spp. was not influenced by the type of peat (Table 5.5).
Table 5.4a Average log reductions of pathogens at pinning and frequency of transfer

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>100:0 Log Reduction(^a)</th>
<th>80:20 Log Reduction(^a)</th>
<th>60:40 Log Reduction(^a)</th>
<th>100:0 Transfer Frequency(^b)</th>
<th>80:20 Transfer Frequency(^b)</th>
<th>60:40 Transfer Frequency(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>3.44</td>
<td>3.17</td>
<td>2.76</td>
<td>0.56</td>
<td>0.56</td>
<td>0.53</td>
</tr>
<tr>
<td><em>Salmonella spp.</em></td>
<td>3.76</td>
<td>3.77</td>
<td>2.99</td>
<td>0.66</td>
<td>0.45</td>
<td>0.53</td>
</tr>
</tbody>
</table>

\(^a\)Based on log reductions from inoculation (Day 0) to pinning (Day 14).
\(^b\)Average frequency of mushroom samples positive for *Listeria* or *Salmonella*.

Table 5.4b Average log reductions of pathogens at harvest and frequency of transfer

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>100:0 Log Reduction(^a)</th>
<th>80:20 Log Reduction(^a)</th>
<th>60:40 Log Reduction(^a)</th>
<th>100:0 Transfer Frequency(^b)</th>
<th>80:20 Transfer Frequency(^b)</th>
<th>60:40 Transfer Frequency(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listeria monocytogenes</em></td>
<td>3.46</td>
<td>4.02</td>
<td>3.18</td>
<td>0.56</td>
<td>0.56</td>
<td>0.53</td>
</tr>
<tr>
<td><em>Salmonella spp.</em></td>
<td>3.60</td>
<td>4.36</td>
<td>3.54</td>
<td>0.66</td>
<td>0.45</td>
<td>0.53</td>
</tr>
</tbody>
</table>

\(^a\)Based on log reductions from inoculation (Day 0) to harvest (Day 20).
\(^b\)Average frequency of mushroom samples positive for *Listeria* or *Salmonella*.

Table 5.5 Qualitative evaluation of peat type and pathogen transfer

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>P-Value(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. monocytogenes</em></td>
<td>0.7513</td>
</tr>
<tr>
<td><em>Salmonella spp.</em></td>
<td>0.7723</td>
</tr>
</tbody>
</table>

\(^a\)Means (n=34) were compared using a Chi-squared test of independence with Fisher’s Exact Test \(P<0.05\).
5.4.4. Antibiotic Assay

Antibiotics from the soil or from the mushrooms were thought to be a potential factor in pathogen reductions, however, no antimicrobial activity was observed for *L. monocytogenes* or *Salmonella* spp. (Table 5.6). Inhibition zones were observed against *Listeria* using the control (nisin) solution, and although it appeared that inhibition was seen against *Salmonella*, upon further inspection it was determined that the “zone” seen was an artifact. Without a chelating agent, the nisin is not effective against Salmonella (Stevens 1991).

Table 5.6 Pathogen inhibition zones

<table>
<thead>
<tr>
<th></th>
<th><em>Listeria</em>&lt;sup&gt;a&lt;/sup&gt; inhibition</th>
<th>Zone diameter (mm)</th>
<th><em>Salmonella</em>&lt;sup&gt;b&lt;/sup&gt; Inhibition</th>
<th>Zone diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nisaplin</td>
<td>Yes</td>
<td>7.0</td>
<td>No</td>
<td>3.5</td>
</tr>
<tr>
<td>Dark peat</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
</tr>
<tr>
<td>Light peat</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
</tr>
<tr>
<td>Small mushroom</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
</tr>
<tr>
<td>Medium mushroom</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
</tr>
<tr>
<td>Large mushroom</td>
<td>No</td>
<td>N/A</td>
<td>No</td>
<td>N/A</td>
</tr>
</tbody>
</table>

<sup>a</sup>*Listeria* used was the same seven-strain cocktail used throughout the paper.  
<sup>b</sup>*Salmonella* used were the same two strains used throughout the paper.
5.5. CONCLUSIONS

Levels of casing soil experimentally inoculated with *L. monocytogenes* and *Salmonella* spp. in the mushroom growing model system decreased significantly for all peat soil types. Pathogen reductions in 100:0 light:dark peat casing soil were significantly greater between inoculation and pinning compared to casing soil containing 40% dark peat. Despite these reductions, pathogen transfer from soil to mushrooms occurred for all soil types and there were not significant differences in transfer rate for each soil type. These findings suggest that the addition of up to 40% dark peat does not introduce a new food safety risk. In fact, the casing step can be considered a natural food safety hurdle since pathogen levels decreased throughout the growing cycle in all combinations of light and dark peat.
CHAPTER 6. EFFECTIVENESS OF PRE-HARVEST SANITIZER IRRIGATION TREATMENTS ON LISTERIA MONOCYTOGENES AND SALMONELLA SPP. LEVELS INOCULATED INTO A MODEL MUSHROOM GROWING SYSTEM

6.1. ABSTRACT

Experiments were conducted to test the effects of select sanitizers on *L. monocytogenes* and *Salmonella* spp. inoculated onto excised mature mushrooms and on mushrooms pins within a model mushroom system. Chlorine dioxide, sodium hypochlorite, hydrogen peroxide, Tsunami 100, and water were evaluated on excised mushroom caps spot-inoculated with *L. monocytogenes* and *Salmonella* spp. Twenty-four hours after inoculation, sanitizers were sprayed onto mushrooms in trays and dried for one hour. Sanitizers were inactivated with a neutralizing broth and mushrooms were enumerated for pathogens. Enrichments were performed to recover injured cells. Log reductions for *L. monocytogenes* and *Salmonella* spp. for control mushrooms were 1.2 and 1.6, respectively. All sanitizer treatments resulted in significantly (P<0.05) greater log reductions compared to the control. For the model mushroom growing system, mushroom pins were spot-inoculated and then irrigated three times between inoculation and harvest with only the third irrigation containing sanitizers. Reductions for both pathogens on control mushrooms were 2.7 log_{10} units. None of the sanitizers tested resulted in significantly greater reductions, as compared to the water control.

6.2. INTRODUCTION

In the previous chapter, inoculation of various combinations of casing soils with *L. monocytogenes* and *Salmonella* spp. in a growing system resulted in partial reduction of the
pathogens. Despite reductions in the levels of pathogens in the casing soils, transfer of pathogens onto emerging mushrooms occurred. This observation warrants investigation into the application of pre-harvest sanitizer treatments to reduce levels of \textit{L. monocytogenes} and/or \textit{Salmonella} spp. on mushrooms. Pathogen reductions will be compared using chlorine dioxide, sodium hypochlorite, hydrogen peroxide, and Tsunami 100 on excised, inoculated mushroom caps, and on growing mushrooms in the model growing system after two water irrigations and a third irrigation treatment supplemented with sanitizers.

\textbf{6.3. MATERIALS AND METHODS}

\textbf{6.3.1. Inoculation procedure for excised mushrooms}

Harvested mushrooms with cap diameters between 18 and 38 mm were obtained from the MTDF. Stems were removed from caps with gloved hands and 30 caps were arranged on each of the seven trays. Cultures of \textit{L. monocytogenes} and \textit{Salmonella} spp. were prepared as described earlier (Chapter 4) except that after re-suspending the cells, the culture cocktail was added to a sterile plastic test tube instead of a spray bottle. Under a biological fume hood, 10 µl of the combined culture was spot inoculated onto the top center of each mushroom cap and allowed to dry for 20 minutes. Mushrooms from six of the seven trays were placed into the Lunaire at 21.5 °C and 99% humidity and incubated for 22 to 26 hours to allow for bacterial attachment. Mushrooms on the single tray that was not incubated were used to enumerate the pathogens immediately after inoculation (Time 0).

\textbf{6.3.2. Treatments for excised mushrooms}

Approximately 24 hours after inoculation, mushrooms on each tray were treated with a sanitizer spray or a control spray containing deionized water only. Sanitizers studied were
sodium hypochlorite (10-15%, Sigma-Aldrich, St. Louis, MO, USA), chlorine dioxide (sodium chlorite acidified with 6% acetic acid, Agrizyme), a commercial fruit and vegetable wash (Tsunami 100, Ecolab, ), and food-grade hydrogen peroxide (35%, Sigma-Aldrich, St. Louis, MO, USA). All sanitizers were prepared by diluting with deionized water until concentrations were at the desired levels (Table 6.1). Chlorine dioxide was generated by acidifying a 5% sodium chlorite solution with a 6.0% acetic acid solution followed by dilution. ClO₂ concentration was measured using the Chlorine Dioxide Test Kit (Hach, Loveland, CO, USA). Sodium hypochlorite free chlorine concentration was measured using a chlorine test kit (Chlorine Free % Total Test Kit, Hach) and pH and ORP measurements were taken with the Series EL2 Portable pH/ORP/Temperature Meter (Mettler Toledo, Columbus, OH, USA). Hydrogen peroxide concentration was measured semi-quantitatively using test strips (Quantofix Peroxide 100, Sigma-aldrich). Tsunami 100 concentration was measured using the Peracid/Peroxide #311 test kit (Ecolab, St. Paul, Minnesota, USA). Desired concentrations for all sanitizers was based on FDA regulations and usage levels at mushroom farms (Table 6.1).

Each sanitizer type was sprayed onto mushrooms placed into separate trays. Sanitizers or water were applied by spraying 20 times, equivalent to 18 ml, evenly onto each of 30 mushrooms in a tray, such that each mushroom was saturated. After each treatment, the tray was placed back into the Lunaire biological safety hood for 1 hour to allow for the sanitizer or water to dry. Sodium hypochlorite treatments were repeated, using three concentrations (50, 100, and 150 ppm) in addition to a water control applied to 15 excised mushroom caps per treatment.
Table 6.1 Sanitizer characteristics measured before application

<table>
<thead>
<tr>
<th>Sanitizer</th>
<th>Desired concentration (ppm)</th>
<th>Actual concentration (ppm)</th>
<th>pH</th>
<th>ORP (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen peroxide</td>
<td>7500</td>
<td>7000-8000</td>
<td>5.10</td>
<td>116</td>
</tr>
<tr>
<td>Tsunami 100</td>
<td>80</td>
<td>80</td>
<td>3.51</td>
<td>206</td>
</tr>
<tr>
<td>Hypochlorous acid</td>
<td>150</td>
<td>~150&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.96</td>
<td>456</td>
</tr>
<tr>
<td>Chlorine dioxide</td>
<td>50</td>
<td>~50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.23</td>
<td>511</td>
</tr>
</tbody>
</table>

<sup>a</sup>Desired concentrations were outside of instrumental detection limits and required dilutions for accurate measurements.

6.3.3. Sampling, enumeration, and enrichments for excised mushrooms

6.3.3.1. Sampling and enumeration

After drying for 1 hour, each mushroom was weighed into a stomacher bag, and Neutralizing Buffer (NB, Difco; Sparks, MD, USA) was added to obtain a 10<sup>1</sup> dilution. NB was chosen because it acts not only as a pH buffer but also as a chlorine inactivator. Each sample was stomached for 2 minutes at 200 rpm, diluted using a 99 ml buffered peptone dilution blank, and spread-plated onto XLD and MOX plates, in duplicate, which were incubated at 35 ºC for 48 hours prior to enumeration.

6.3.3.2. Enrichments

Enrichments for <i>L. monocytogenes</i> and <i>Salmonella</i> spp. were carried out according to procedures described in Chapter 3; samples that were positive after direct plating were not enriched further. From each sample homogenate, 1 ml aliquots were distributed into UVM and lactose primary enrichment broths and incubated at 35 ± 2ºC for 24 hours. Mushroom samples that were positive for <i>L. monocytogenes</i> and/or <i>Salmonella</i> spp. after direct plating, primary enrichments, and/or secondary enrichments, and confirmed using tests described previously were
counted as positives for the respective bacterium. The detection limit for countable plates was 1.30 log$_{10}$ CFU/gram.

6.3.4. *Preparation and inoculation procedure for pins in a model mushroom growing system*

Substrate and casing materials were obtained from the MTDF (Crop # 1103). Containers were prepared according to procedures described in Chapter 3, except that only casing soil containing 100 % light peat and 2% CI was used and the soil was not inoculated with *L. monocytogenes* or *Salmonella* spp.. Containers were grown according to procedures described previously with regular irrigation and rotation within the chamber for the initial 13 days. Containers with at least one mushroom pin with a diameter between 5.0 and 18 mm were chosen, and mushroom pins were spot-inoculated as described previously. Each inoculated cap was marked with a blue permanent marker (Sharpie, Newell Rubbermaid, Atlanta, GA, USA) adjacent to the inoculation area to distinguish them from uninoculated fruiting bodies that might emerge at a later time.

6.3.5. *Treatments for mushrooms in a model mushroom growing system*

After inoculation, mushroom containers were returned to the Lunaire biological safety hood for 24 hours to allow for attachment, and then watered with tap water. Watering was repeated 24 hours later. After another additional 24 hours, sanitizer treatments incorporated into the third watering were applied. This treatment scheme was chosen to simulated industry practices to apply pre-harvest sanitizers where sanitizers, if used, are typically incorporated into the last watering just prior to harvest.
To assure sufficient sample size, 5 groups of 10 containers were selected so that each group had a total of at least 30 inoculated mushrooms. Each group of 10 containers therefore received a different sanitizer treatment or the control. Sanitizers were applied to each separate container by using 5 sprays of approximately 4.5 ml evenly distributed to the mushrooms. This volume was chosen to allow the mushrooms to become completely saturated after application. The containers were then returned to the Lunaire chamber for 1 hour ± 10 minutes to allow for surface drying before processing. Sanitizer concentrations applied to the inoculated pins in the model growing system were the same as described previously.

6.3.6. **Sampling, enumeration, and enrichments for mushrooms in a model growing system**

**6.3.6.1. Sampling and enumeration**

Mushrooms were sampled 4 times: immediately after inoculation (0 hours); before the first watering (24 hours); before the third watering which contained sanitizers (72 hours); and then 1 hour after the treatments were applied.

For sampling, containers were moved to a biological fume hood where each inoculated mushroom was cut using a sterile scalpel as described (Chapter 5). Inoculated mushrooms from the same container were weighed into a stomacher bag and NB was added at a 1:2 dilution. Bags were stomached for 2 minutes at 200 rpm and 0.1 ml of each sample was spread-plated onto XLD and MOX agar, in duplicate, and incubated at 35 ± 2°C for 48 hours.

**6.3.6.2. Enrichments**

Enrichments were performed according to procedures described previously, where only negative samples were transferred to the secondary enrichment broths.
6.3.7. Statistical analysis

Data was analyzed using a one-way analysis of variance (ANOVA) and using Tukey’s Honestly Significant Difference (Tukey’s HSD), and differences between bacterial counts in the types of peat were determined based on a significance level of \( P<0.05 \) (SAS 9.1.3, Cary, NC).

Qualitative data for presence/absence of pathogens on mushrooms in relation to the sanitizer treatment was analyzed using Chi-squared test for independence where \( P<0.05 \) (SAS 9.1.3, Cary, NC).

6.4. RESULTS AND DISCUSSION

6.4.1. Comparison of treatments for excised mushrooms

Levels of *L. monocytogenes* and *Salmonella* spp. inoculated onto excised mature mushrooms decreased by less than 1 log unit after 24 hours of attachment time (Table 6.2). The control treatment resulted in a 1.2 and 1.6-log reduction in *L. monocytogenes* and *Salmonella* spp., respectively, which was the smallest log reduction compared to the other treatments (Table 6.3). Treatment reductions were based on pathogens present 24 hours after inoculation. Greatest log reductions were observed when sodium hypochlorite was applied at 150 ppm, with 3.4 and 3.3-log reductions in *L. monocytogenes* and *Salmonella* spp., respectively. This treatment resulted in significantly lower pathogen counts compared to the other sanitizers. Other treatments had intermediate effects. For *L. monocytogenes*, chlorine dioxide, hydrogen peroxide, and Tsunami 100 all resulted in pathogen counts significantly lower than those recovered from the control. For *Salmonella* spp., hydrogen peroxide and Tsunami 100 resulted in counts significantly lower than the control. Sodium hypochlorite at 100 and 150 ppm resulted in
significant reductions for both pathogens, while all three sodium hypochlorite concentrations were effective for *L. monocytogenes* counts (Table 6.4). In qualitative analyses, overall presence or absence of *L. monocytogenes* and *Salmonella* spp. were influenced by the treatment used (Table 6.5).

### Table 6.2 Recovery of pathogens inoculated onto excised mushroom caps

<table>
<thead>
<tr>
<th>Time after inoculation&lt;sup&gt;a&lt;/sup&gt; (h)</th>
<th><em>Listeria monocytogenes</em></th>
<th><em>Salmonella</em> spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Counts on MOX log(CFU/g)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Counts on XLD log(CFU/g)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0</td>
<td>5.2 ± 0.10</td>
<td>5.7 ± 0.29</td>
</tr>
<tr>
<td>24</td>
<td>4.6 ± 0.21</td>
<td>5.0 ± 0.31</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean inoculum populations for *L. monocytogenes* and *Salmonella* spp. were 6.3 ± 0.61, and 6.6 ± 0.46 CFU, respectively.

<sup>b</sup>Mean (n=4) ± standard deviation.
Table 6.3  Efficacy of sanitizers applied to excised mushrooms inoculated with *Listeria monocytogenes* and *Salmonella* spp.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Listeria monocytogenes</th>
<th>Salomonella spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of positive samples (n=30)</td>
<td>Counts on MOX log(CFU/g)</td>
</tr>
<tr>
<td>Control (water)</td>
<td>30</td>
<td>3.4 ± 0.58 A</td>
</tr>
<tr>
<td>Chlorine dioxide (50 ppm)</td>
<td>28</td>
<td>2.7 ± 1.37 B</td>
</tr>
<tr>
<td>Tsunami 100 (80 ppm)</td>
<td>24</td>
<td>2.1 ± 1.46 B</td>
</tr>
<tr>
<td>Hydrogen peroxide (7500 ppm)</td>
<td>26</td>
<td>2.7 ± 1.43 B</td>
</tr>
<tr>
<td>Hypochlorous acid (150 ppm)</td>
<td>18</td>
<td>1.2 ± 1.35 C</td>
</tr>
</tbody>
</table>

*a*Mushrooms positive for respective pathogen after direct plating, primary, and secondary enrichments.

*b*Means (n=30) with different letters are significantly different within column rows (*P*<0.05).

*c*Based on mushrooms enumerated 24 hours after inoculation.
Table 6.4 Efficacy of different concentrations of hypochlorous acid applied to excised mushrooms inoculated with *L. monocytogenes* and *Salmonella* spp.

<table>
<thead>
<tr>
<th>Treatment</th>
<th><em>Listeria monocytogenes</em></th>
<th></th>
<th><em>Salmonella</em> spp.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Counts on MOX log(CFU/g)</td>
<td>Log reduction</td>
<td>Counts on XLD log(CFU/g)</td>
<td>Log reduction</td>
</tr>
<tr>
<td>Control (water)</td>
<td>3.9 ± 0.15 A</td>
<td>2.4</td>
<td>4.0 ± 0.08 A</td>
<td>2.2</td>
</tr>
<tr>
<td>Hypochlorous acid (50 ppm)</td>
<td>3.8 ± 0.12 B</td>
<td>2.5</td>
<td>3.9 ± 0.12 AB</td>
<td>2.3</td>
</tr>
<tr>
<td>Hypochlorous acid (100 ppm)</td>
<td>3.8 ± 0.13 B</td>
<td>2.5</td>
<td>3.8 ± 0.37 B</td>
<td>2.4</td>
</tr>
<tr>
<td>Hypochlorous acid (150 ppm)</td>
<td>3.6 ± 0.14 C</td>
<td>2.7</td>
<td>3.6 ± 0.14 C</td>
<td>2.6</td>
</tr>
</tbody>
</table>

*a*Means (n=15) with different letters are significantly different within column rows (*P*<0.05).

*b*Based on mushrooms enumerated 24 hours after inoculation (6.3 ±0.06 log CFU/g for *L. monocytogenes* and 6.2 ± 0.08 log CFU/g for *Salmonella* spp.).

Table 6.5 Qualitative evaluation of sanitizers and presence of pathogens on excised mature mushrooms

<table>
<thead>
<tr>
<th>Pathogen</th>
<th>P-Value(^a)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. monocytogenes</em></td>
<td>&lt;0.0001(^*)</td>
</tr>
<tr>
<td><em>Salmonella</em> spp.</td>
<td>0.009(^*)</td>
</tr>
</tbody>
</table>

*a*Means (n=30) were compared using a Chi-squared test of independence with Fisher’s Exact Test where an asterisk (*) indicates significance (*P*<0.05).
6.4.2. *Comparison of treatments for mushrooms in a model growing system*

Sanitizers were not effective, however, in the model mushroom growing system when compared to the control water treatment (Table 6.5). Pathogen levels decreased slightly after initial inoculation and after two irrigations with tap water (Table 6.6). A 2.7-log reduction was observed in both pathogens after three irrigations with no sanitizers added to the water. The sanitizer treatments also resulted in pathogen reductions ranging from 2.5 to 3.9 logs. However, reductions were not significantly different from the control. In qualitative analyses, overall presence or absence of *L. monocytogenes* and *Salmonella* spp. was not influenced by the treatment used.

### Table 6.6 Recovery of pathogens inoculated onto growing mushroom pins

<table>
<thead>
<tr>
<th>Time after inoculation&lt;sup&gt;a&lt;/sup&gt; (h)</th>
<th><em>Listeria monocytogenes</em></th>
<th><em>Salmonella</em> spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Counts on MOX log(CFU/g)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Counts on XLD log(CFU/g)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>0</td>
<td>6.1 ± 0.08</td>
<td>5.8 ± 0.19</td>
</tr>
<tr>
<td>24</td>
<td>5.9 ± 0.10</td>
<td>5.6 ± 0.26</td>
</tr>
<tr>
<td>72</td>
<td>6.0 ± 0.92</td>
<td>5.5 ± 0.15</td>
</tr>
</tbody>
</table>

<sup>a</sup>Mean inoculum populations for *L. monocytogenes* and *Salmonella* spp. were 6.9 ± 0.36, and 6.8 ± 0.93 CFU, respectively.

<sup>b</sup>Mean (n=4) ± standard deviation.
Table 6.7 Efficacy of sanitizers applied as a third irrigation treatment to a model mushroom growing system inoculated with *Listeria monocytogenes* and *Salmonella* spp.

<table>
<thead>
<tr>
<th>Treatment</th>
<th><em>Listeria monocytogenes</em></th>
<th><em>Salmonella</em> spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of positive samples</td>
<td>Counts on MOX</td>
</tr>
<tr>
<td></td>
<td>(n=10)</td>
<td>log(CFU/g)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Log reduction</td>
</tr>
<tr>
<td>Control (water)</td>
<td>10</td>
<td>3.3 ± 1.05 AB</td>
</tr>
<tr>
<td>Chlorine dioxide (50 ppm)</td>
<td>10</td>
<td>3.2 ± 1.01 AB</td>
</tr>
<tr>
<td>Tsunami 100 (80 ppm)</td>
<td>10</td>
<td>3.3 ± 1.21 AB</td>
</tr>
<tr>
<td>Hydrogen peroxide (7500 ppm)</td>
<td>10</td>
<td>3.5 ± 0.65 A</td>
</tr>
<tr>
<td>Hypochlorous acid (150 ppm)</td>
<td>10</td>
<td>2.6 ± 1.22 B</td>
</tr>
</tbody>
</table>

*a*Containers with mushrooms positive for respective pathogen after direct plating, primary, and secondary enrichments.

*b*Means (n=10) with different letters are significantly different within column rows (*P*<0.05).

*c*Based on mushrooms enumerated 24 hours after inoculation.

### 6.5. CONCLUSIONS

All sanitizers were effective in reducing levels of *L. monocytogenes* and *Salmonella* spp. on inoculated excised mushroom caps. However, results from the model mushroom growing system indicated that none were more effective than the 2.7 logs reduction achieved by the water control. Since log reductions observed in the control irrigation treatment were not different than when sanitizers were added, decreases in pathogens between pinning and harvesting may be contributed to the properties of the mushrooms themselves than the external treatments.
CHAPTER 7. SUMMARY AND CONCLUSIONS

The first objective (Chapter 3) of this research was to compare levels of indigenous microflora in light and dark peat casing soils. Results from these experiments showed that the indigenous microbial populations were lower in dark peat compared to light peat. When light peat is combined with dark peat, levels of indigenous microflora were highest in the combinations that contain lower levels of dark peat.

The second objective (Chapter 4) was to determine the fate of human pathogens inoculated into both light and dark peat casing soils. Over a 29-day period, levels of *L. monocytogenes* and *Salmonella* spp. decreased significantly in light peat and in dark peat. By the final sampling day, log reductions of *L. monocytogenes* in light, Harte dark, and Sylvan dark were 5.17, 5.34, and 5.44 logs, respectively. Log reductions of *Salmonella* spp. were 4.72, 3.55, and 3.48 logs, respectively. It was also shown that human pathogens remained unchanged in autoclaved light and dark peats soils, which confirms earlier studies using light peat only. These findings suggested that heat-treating peat may introduce new food safety risks.

The third objective (Chapter 5) was to determine the fate of human pathogens inoculated into casing soils in a model mushroom growing system with different combinations of light and dark peat. Results of this study showed that levels of *L. monocytogenes* and *Salmonella* spp. significantly decreased over the course of a growing cycle, regardless of the peat combination. Furthermore, it was shown that pathogens can transfer from contaminated soil to the emerging mushrooms.

The fourth objective (Chapter 6) was to compare effectiveness of sanitizers on human pathogens inoculated onto mushrooms. When inoculated, excised mushrooms were treated with sanitizers, all sanitizers reduced pathogen levels, as compared the control. However, in a model
Growing system, the use of sanitizers in the third irrigation treatment did not provide any additional antibacterial effect, as compared to the control. Therefore, addition of sanitizers in the third irrigation treatment cannot be recommended as a pre-harvest intervention to reduce levels of human pathogens.

Overall, this research showed that the addition of up to 40% dark peat does not impose a food safety risk for human pathogens that may contaminate the casing soil. Pathogens were reduced to lower levels between casing and harvest, and this reduction was not affected by peat casing soil type. Therefore, the casing step itself can be considered a food safety intervention, providing a 3 to 4-log reduction, regardless of peat soil type. Irrigation with only water resulted in a pathogen reduction on mushrooms of 2.7 logs. Therefore, commercial growing practices have, in effect, two valuable food safety hurdles built into the system.

There is still some risk associated with contamination of fresh mushrooms. As with any food processing facility, one of the major preventative measures that can be taken to minimize contamination is to keep raw materials separate from materials that have been pasteurized, and to have designated areas for both. This approach is outlined in the MGAP program, along with employee hygiene and food safety practices to prevent contamination, as well as supplier reliability, field packing and protection of harvested mushrooms, and cleaning and sanitizing food contact surfaces. Although natural pathogen reductions do occur, the importance of following these industry-wide standards must be stressed in order to prevent contamination of casing soils and mushrooms.
CHAPTER 8. SUGGESTIONS FOR FUTURE STUDIES

It would be beneficial to further explore the interactions in the peat casing soils, and investigate the possible competition that may be occurring in the soils or on the mushrooms themselves. The presence of Agaricus bisporus did not appear to have an effect on pathogen levels, as compared to the inoculation studies without Agaricus. Also with respect to mechanisms behind pathogen suppression, investigating the microbial ecology using molecular techniques may give insight into some of the unculturable bacteria present in the soils. A molecular approach also would provide information about the relative species diversity within the peat casing soils.

Levels of nutrients over the course of a growing period could be followed to see if there is more nutrient depletion in the soils with only light peat, as compared to those with high levels of dark peat. In the presence of Agaricus bisporus, dark peats may provide more nutrients for pathogens to grow.

Autoclaving peat soils is not practical in industry, however, peat soils may be steam-treated at lower temperatures. It would be interesting to investigate the effects of steam treatments at various time/temperature combinations on levels of indigenous microflora in the soils. In addition, steam-treated soils could be inoculated with L. monocytogenes and Salmonella spp. and used in the model mushroom growing system described in previous chapters, where pathogen levels could be monitored over time. Peat soils may also be inoculated with L. monocytogenes and Salmonella spp. prior to steam treatments to possibly determine a time/temperature combination that effectively eliminates pathogens but maintains enough of the indigenous microflora so that the suppressive properties of the casing soil do not change.
Sanitizers used in this study were formulated to levels commonly used in the fresh produce industry or have been used previously on mushrooms. However, comparable results could be obtained if all sanitizers were evaluated at the same concentrations, in order to compare effectiveness. Further research studying the properties of growing mushrooms between pinning and harvest that result in pathogen reduction without sanitizers would be beneficial. It is unclear if mushrooms support the growth of *L. monocytogenes* and *Salmonella* spp., so it would be interesting to investigate the properties of growing mushrooms, such as indigenous microflora, metabolites produced, etc. Although an antimicrobial assay was conducted in this research, a more in-depth look at organisms producing antimicrobials in the soil, as well as secondary metabolites produced by the mushrooms, would be beneficial in understanding the suppressive effects observed with the pathogens.

Ultimately, steps to reduce contamination of fresh mushrooms must be followed throughout the pre-harvest period and also during post-harvest and storage. Contamination during the slicing and/or packing operations can occur because of human contact as well as numerous food contact surfaces. These operations would be the next logical step to explore for contamination sources of fresh mushrooms. An environmental monitoring plan in a slicing facility may give a clearer picture of possible sources of *L. monocytogenes* and *Salmonella* spp. contamination. Also, it may be of interest to explore *Staphylococcus aureus* contamination during the harvesting and packaging steps, since post-harvest food safety practices, packaging, and storage temperatures can all affect the growth of *S. aureus* and enterotoxin production (Martin and Beelman 1995). Perhaps this step of the process would be ideal for introducing a food safety intervention, such as an additional cleaning step, in order to reduce contamination of sliced mushrooms with pathogens.
### Appendix

<table>
<thead>
<tr>
<th>MTDF</th>
<th>Laboratory</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate and casing in wooden trays, substrate packed tightly and casing layered on top</td>
<td>Substrate (350 g) packed tightly into deli containers, casing (200 g) layered on top</td>
<td>Many containers could fit into the chamber, and size made it convenient for destructive sampling.</td>
</tr>
<tr>
<td>Humidity is 100% for first 6 days of cycle, then decreased to 92%</td>
<td>Humidity is set as high as possible &amp; containers are wrapped in garbage bags, removed on Day 9</td>
<td>Humidity is kept in the containers, removal lowers humidity slightly and increases air flow</td>
</tr>
<tr>
<td>Temperature is at 24°C for 6 days, then lowered to 20.1 ºC</td>
<td>Temperature is at 24°C for 9 days, then lowered to 20.1 ºC</td>
<td>Laboratory crop is a few days behind MTDF crop</td>
</tr>
<tr>
<td>Watering is done at growers discretion; around every other day until initials develop</td>
<td>Watering done every day for the first 9 days since containers are small they dry out quicker</td>
<td>Although watering intervals vary in a practical setting, generally it was consistent in the laboratory</td>
</tr>
<tr>
<td>Amount of watering is one even layer of the watering hose over each bed</td>
<td>Watering is done with calibrated squirt bottle, 7.2 ml (8 squirts)</td>
<td>Water is sprayed from approx. 6” away to cover soil in each container</td>
</tr>
<tr>
<td>Harvest occurs when stems are flat and caps flatten out</td>
<td>Harvest occurs when stems are flat and caps flatten out</td>
<td>Harvested with maturity index between 2 and 6</td>
</tr>
</tbody>
</table>
Containers before (left) and after (right) watering.
REFERENCES


Cutter, C. N. and Miller, B. J. 2005. Incorporation of nisin into a collagen film: Retains activity against *Listeria monocytogenes* and *Brochothrix thermophacta* associated with a Ready-To-Eat meat product. Department of Food Science.


Royse, Daniel J. and Beelman, Robert B. 2007. Six Steps to Mushroom Farming. College of Agricultural Sciences Agricultural Research and Cooperative Extension. The Pennsylvania State University, University Park, PA, USA.


