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Persistence and internalization of Listeria monocytogenes in romaine lettuce, Lactuca sativa var. longifolia

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PERSISTENCE AND INTERNALIZATION OF *LISTERIA MONOCYTOGENES* IN ROMAINE LETTUCE, *LACTUCA SATIVA* VAR. LONGIFOLIA

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For Dad, my personal hero

For Mum, the strongest woman I know

For Aaron, the best partner in crime a girl could ever ask for

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ABSTRACT

Shenoy, Archana G. M.S., Purdue University, May 2015. Persistence and Internalization of *Listeria monocytogenes* in Romaine Lettuce, *Lactuca sativa* var. longifolia. Major Professor: Haley Oliver and Amanda Deering.

Listeria monocytogenes, has been implicated in a number of outbreaks involving fresh produce. While no *L. monocytogenes* outbreaks have been linked to romaine lettuce, the number of lettuce recalls specific to *L. monocytogenes* is increasing. Understanding the potential of persistence and internalization of *L. monocytogenes* on and within romaine lettuce will aid in determining food safety risk. Persistence of three *L. monocytogenes* strains on three romaine lettuce cultivars was assessed independently by inoculating seeds in 25 ml of 8 log CFU/ml for 30 minutes. Seeds were grown on two soil types (i.e. standard potting mix, Indiana top soil) or sterile soft-top agar for up to 60 days. Average CFU/g of *L. monocytogenes* retained on seeds or persisting on growing plants was calculated from a total of 5 replicates per harvest day. Plants grown on sterile soft-top agar maintained between 4.4 to 7.8 log CFU/g *L. monocytogenes* after a 60 day period, while pathogen levels dropped below the limit of detection (2 log CFU/g) by Day 18 in 75% Indiana top soil, and by Day 45 in commercial potting mix. This suggests that soil microflora may impede pathogen persistence. *L. monocytogenes* strain differences and the presence of a clay coating on seeds were not

factors that affected persistence. Cultivar differences, however, potentially influenced *L. monocytogenes* growth and survival. For internalization studies, seeds were inoculated with a *L. monocytogenes* strain constitutively expressing green fluorescent protein (GFP). Three plants were fixed, paraffin embedded, and sectioned; localization was studied using standard immunohistochemistry techniques. A total of 539 *L. monocytogenes* cells were internalized in all major tissue types of the hypocotyl with the majority localizing in the pith followed by cortex, xylem, phloem and epidermis. The presence of the bacterium in the plant vasculature indicates its potential to be transported throughout the plant system and reside within edible tissue. The significance of these findings is that romaine lettuce can support growth and internalization of *L. monocytogenes*, which could serve as a vehicle for *L. monocytogenes* transmission to consumers. Additionally, seeds from 16 cultivars of romaine lettuce and one cultivar of radicchio were examined as a potential source of contamination in a 2014 commercial recall of bagged salad products. Only a single seed, out of 1,700 tested, was found to be contaminated using PCR based methods on isolates recovered. These data indicate that while it is possible that the seed could be a source of potential *L. monocytogenes* contamination, it is unlikely that the seeds of the tested cultivars were the source of bagged salad recalls.

CHAPTER 1. LITERATURE REVIEW

1.1 *Listeria monocytogenes*

1.1.1 Introduction to *Listeria monocytogenes*

Listeria monocytogenes has gone from being a relatively unknown pathogen 30 years ago to being one of the major food safety concerns in the food industry. *L. monocytogenes* is one of 15 species in the *Listeria* genus with the others including *L. innocua, L. seeligeri, L. welshimeri, L. ivanovii* and *L. grayi* (Vazquez-Boland et al., 2001). More recently, another nine species were added and these include *L. rocourtiae* (Leclercq et al., 2010), *L. weihenstephanensis* (Lang Halter et al., 2013), *L. fleischmannii* (Bertsch et al., 2013), *L. marthii* (Graves et al., 2010), *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. riparia*, and *L. grandensis* (den Bakker et al., 2014). Of these, only *L. monocytogenes* and *L. ivanovii* are considered to be pathogenic. The former being pathogenic to both humans and animals while the latter is rarely found to cause pathogenesis in humans (Liu, 2006).

L. monocytogenes is a Gram-positive, facultative anaerobe, non-spore forming, rod-shaped bacteria that is usually found to be within 1 – 1.5 µm in length (Liu, 2006). It is found ubiquitously in the environment and has been isolated from ground water, soil

and silage (Gray et al., 2006). *L. monocytogenes* is typically found as a saprophyte, feeding off dead and decaying matter (Freitag et al., 2009). However, it is a hardy bacterium, and is able to tolerate various environmental stressors. *L. monocytogenes* not only has the ability to survive a wide temperature range of 0 to 45°C, it can also withstand salt concentrations up to 10% sodium chloride and even a pH range of between pH 4.4 to pH 9.4 (FAO/WHO, 2004, Pearson and Martha, 1990). This propensity towards being able to adapt to its environment is likely what makes *L. monocytogenes* so successful in its ability to survive and replicate in human host cells following ingestion (Chaturongakul et al., 2008).

There is considerable diversity among strains of *L. monocytogenes* with 13 different serotypes identified. Serotypes are further classified into lineages. Serotypes 1/2b, 3b, 4b, 4d, 4e, and 7 are included in Lineage I. Lineage II contains serotypes 1/2a, 1/2c, 3a, and 3c while Lineage III has serotypes 4a and 4c (Cossart, 2011). An additional lineage, Lineage IV, has recently been suggested. The first strain to be classified as Lineage IV, strain FSL J1-208, has a small genome in addition to the presence of a possible virulence plasmid (den Bakker et al., 2012). Strains contained within lineages, however, have different virulence capacities. Strains in Lineage I, notably of 1/2b and 4b serotypes, often are associated with clinical listeriosis cases while Lineage II isolates, notably serotype 1/2a, are more often derived from food sources (Liu, 2006, Oliver et al., 2010). Lineages III and IV are more common in animal cases (den Bakker et al., 2012).

1.1.2 Listeriosis

Listeriosis is the bacterial infection caused by the invasion of *Listeria monocytogenes* into mammalian cells (Gray et al., 2006). Mortality rates have been shown to be approximately 16-20% depending on the source cited (Mead et al., 1999, Scallan et al., 2011). *L. monocytogenes* accounts for nearly 1600 cases of listeriosis, 1455 hospitalizations and 255 deaths on an annual basis in the United States (Scallan et al., 2011). This infection can manifest in two different ways. In otherwise healthy individuals, the infection can cause febrile gastroenteritis and result in symptoms such as fever, headache, diarrhea, nausea, vomiting, abdominal pain and arthromyalgia (Ooi and Lorber, 2005, Sim et al., 2002). The more invasive form of the disease usually manifests itself in individuals who are immunocompromised, especially if their T-cell mediated immunity is somehow suppressed (Farber and Peterkin, 1991). Individuals in this group usually include the elderly, young children, pregnant mothers and those with preexisting conditions such as diabetes mellitus or AIDS (Swaminathan and Gerner-Smidt, 2007). *L. monocytogenes* is able to cross 3 vital barriers in the human system including the intestinal, placental, and blood brain barrier (Lecuit, 2005). Crossing of the intestinal barrier results in gastroenteritis type symptoms as discussed above. Once through the mucosa of the gastrointestinal tract, the pathogen can spread via the bloodstream to the spleen and liver (Vazquez-Boland et al., 2001). Ability of *L. monocytogenes* to cross the blood brain barrier results in infection of the central nervous system and can manifest as meningitis, meningoencephalitis, and

rombenchephalitis among others (Drevets et al., 2004). In maternofetal listeriosis, the mother may either be asymptomatic, experience flu-like symptoms, or gastrointestinal problems. However, once the pathogen has crossed the placental barrier, the infant may experience a variety of disease manifestations from meningitis to septicemia to fetal death (Rocourt, 1996). Pregnant women may also experience spontaneous abortions as a result of the infection (Kaur et al., 2007).

The intracellular growth and spread of *L. monocytogenes* has been extensively studied (Cossart and Toledo-Arana, 2008). A variety of gene products are included in the mechanism by which the pathogen moves from one cell to another (Gray et al., 2006). In general, *L. monocytogenes* expresses cell surface proteins Internalin A and Internalin B which bind to cell surface receptors, E-cadherin and Met respectively, on intestinal epithelial cells and induce phagocytosis. Listeriolysin O (LLO), a pore forming toxin, aids in escape from the vacuole. Once in the cellular cytosol, ActA induces actin assembly and enables directional propulsion of *L. monocytogenes* through the cytoplasm and into the neighboring cell (Pamer, 2004). Finally, to enable lysis of the secondary vacuole formed in the new cell, a phosphatidylcholine-specific phospholipase, PlcB, is recruited in conjunction with LLO. This direct cell to cell spread of the pathogen allows it to evade host defense systems and detection more easily (Cossart and Toledo-Arana, 2008). Many of the proteins described above are expressed by genes that are controlled by a key transcriptional activator, PrfA. Strains with mutated or nonfunctional PrfA show a reduction or loss in virulence (Gray et al., 2006).

1.1.3 *Listeria monocytogenes* and food

Foodborne transmission accounts for 99% of human listeriosis infections (Mead et al., 1999). In a 2003 risk assessment of relative risk to public health, the U.S. Food and Drug administration (FDA) found the following categories of food to be high risk in a case per annum basis: deli meat, high fat and other dairy products, and unreheated frankfurters. Soft unripened cheese, cooked ready-to-eat (RTE) crustaceans and smoked fish were all considered to be in the moderate risk category. Various other types of cheese, dry fermented sausages and most notably, produce related food items, were listed in the low risk category. Produce related food items typically include products such as fruits, vegetables and deli-type salads (FDA, 2003b). Between the years 2003 to 2011, *L. monocytogenes* accounted for roughly 18% of all microbiologically contaminated product recalls (Dey et al., 2013).

While produce items have historically been considered low risk with regards to *L. monocytogenes*, the number of foodborne illness outbreaks linked to fresh produce has been increasing (Sivapalasingam et al., 2004). Less than 1% of documented foodborne outbreaks in the United States recorded during the 1970s were due to produce (DeWaal and Bhuiya, 2009, Doyle, 2008). However, in the period between 1990 and 2005, that number increased to an estimated 13% (Doyle, 2008, Sivapalasingam et al., 2004). A variety of factors could have contributed to this recent rise including the increased per capita consumption of fresh vegetables and fruits, larger volumes of produce being shipped from centralized locations to further areas, an increase in the at-risk population

consuming produce, and even improvements in surveillance of outbreak investigations (FDA, 2001). Within the past five years, there have been a few outbreaks of *L. monocytogenes* related to produce. In 2010, *L. monocytogenes* was isolated from diced celery that was used as an ingredient in chicken salad and served at various Texas hospitals. The outbreak spanned a duration of 7 months and involved 5 hospitals, with a total of 10 confirmed cases. All cases involved were elderly people who were already admitted at a hospital which could help explain the 50% mortality rate in this case (Gaul et al., 2013). The next outbreak occurred the very following year in 2011. This outbreak, associated with Rocky Ford cantaloupes grown at Jensen Farms, had far-reaching effects. A total of 147 people from 28 states were infected. A total of 33 deaths were associated with the outbreak, including a mother who miscarried due to the infection. This made the 2011 cantaloupe outbreak the second most deadly outbreak in U.S history (FDA, 2012). In 2014, two smaller outbreaks involving sprouts and apples were reported. In the outbreak involving Granny Smith and Gala apples, the most recent count indicated 32 cases over 11 states (CDC, 2014).

L. monocytogenes has been shown to have the ability to grow on a variety of other types of produce such as asparagus (Rodríguez et al., 2000), broccoli (Berrang et al., 1989), and cabbage (Kallander et al., 1991) even at refrigeration temperatures. This, combined with the fact that the pathogen can be found in the home, food processing environments, and retail environments (FDA, 2003b) is a reason to promote good sanitation practices and routine testing in food environments. *L. monocytogenes* infectious dose has been estimated to be high based on data from epidemic and

sporadic cases (Vazquez-Boland et al., 2001). However, since there is no confirmation of a known infectious dose for *L. monocytogenes* and development of listeriosis is dependent on the health of the individual (Harris et al., 2003), any source of contamination of the pathogen can result in serious consequences. This is especially true as most fresh produce is minimally processed and consumed raw (Olaimat and Holley, 2012).

1.2 Plant-microbe interactions

1.2.1 Sources of produce contamination

There are a variety of contamination sources when it comes to produce, all the way from "farm to fork". These are usually split into pre- and post-harvest sources of contamination. Post-harvest contamination can occur from any of the following (this list is not considered exhaustive): harvesting equipment, worker or consumer handling, transport containers, factory mechanical processing equipment, and even inadequate storage temperatures or improper packaging (FDA, 2001). Only pre-harvest contamination sources will be discussed in detail as it is more applicable to the work presented here.

Common sources of contamination in the pre-harvest period include soil, irrigation water, improperly composted manure, and both wild and domestic animals. Studies have shown that water can not only act as a source of pathogens for produce,

but that it can also act as a vehicle to introduce pathogens into the environment (Strawn et al., 2013) via flooding or surface runoff from animal pastures (Berger et al., 2010, Brandl, 2006). Even contaminated irrigation water can have differing likelihoods of pathogen transmission depending on the method of dispersal. Pathogen transmission was found to be higher from contaminated water when overhead sprinklers were used versus drip irrigation (Mitra et al., 2009). Yet another study showed that 90% of lettuce plants were contaminated when spray irrigated versus 19% of plants that were contaminated when surface irrigated. Both irrigation methods used water contaminated with the same 7 log CFU/ml of *Escherichia coli* O157:H7 (Solomon et al., 2002).

A variety of human pathogens make their home in natural environments such as soil. This includes *L. monocytogenes, Clostridium botulinum, and Bacillus cereus* among others. However, the list of pathogens found in soil undoubtedly expands once manure is added to it (Whipps et al., 2008). Application of raw or inadequately composted animal manure can contribute to the increase or addition of pathogenic microorganisms (Berger et al., 2010). In fact, farms that use animal manure have a higher propensity towards contamination of produce (Doyle, 2008). *L. monocytogenes* has been isolated from cattle (Nightingale et al., 2004), swine (Yokoyama et al., 2005), deer, and even bird feces (Weis and Seeliger, 1975). Both roaming wild or domestic animals could easily defecate in the vicinity of produce fields and contribute to contamination. In addition, certain pathogens are able to survive for months in soil (Doyle, 2008). *L. monocytogenes* has been shown to survive in soil for a period of up to 295 days (Welshimer, 1960). A recent study has shown that up to 17.5% of produce fields were found to be

contaminated with *L. monocytogenes* with irrigation, soil cultivation and wildlife sightings in the area all contributing to an increased likelihood in finding a positive field (Strawn et al., 2013). With certain produce that is grown close to the ground, like lettuce, there is a good possibility that it can come into contact with the soil and become contaminated.

With the potential for contamination so high with regard to pre-harvest conditions, it is expected that the Food and Drug Administration (FDA) would develop regulatory protocols to establish standards for produce safety. These regulations are required by the Food Safety Modernization Act (FSMA) of 2011. The Produce Rule has various specifications that commercial farmers must follow including: microbial standards in water quality testing, types of treatment or applications of soil amendments of animal origin, and worker health and hygiene training. These regulations, however, are still in their revisions and comments phase and do not yet apply (FDA, 2011). Additionally, the FDA has published guidelines to aid the industry in minimizing microbial contamination in produce through the use of Good Agricultural Practices (GAPs). GAPs involve the use of treatments such as proper composting to reduce the potential pathogenic populations in manure or organic materials, the physical separation of manure treatment and storage areas from produce processing or handling areas, and even allowing for maximum possible time between the application of manure and harvesting of the crop (FDA, 1998).

1.2.2 Attachment, growth, and survival of pathogenic bacteria on plants

While growth and survival of pathogens in an animal host provides its own set of challenges, the plant phyllosphere (habitat for microorganisms in the above-ground plant portion) also lends itself to harsh, fluctuating conditions (Berger et al., 2010). Environmental stressors include UV exposure, poor nutritional availability in certain plants, variations in osmotic or temperature conditions, and even plant defenses (Berger et al., 2010, Doyle, 2008, Lindow and Brandl, 2003, Whipps et al., 2008). While pathogens are not necessarily a normal part of the phyllosphere, various produce associated foodborne illness outbreaks, as discussed above, have shown that they are clearly capable of survival in such an environment. Survival of these pathogens against environmental stressors is probably dependent on their ability to colonize plant microsites in which conditions are protective and more favorable. Various physicochemical conditions and variation in leaf topography allows these microsites to exist (Brandl, 2006).

Understanding how pathogens may attach to produce is important since attachment is the first step in a bacterium being able to colonize, or potentially even internalize, in the edible portions of the plant. Information on attachment mechanisms could even be used to develop prevention strategies. Of produce associated outbreaks linked to a known bacterial pathogen between the years of 1973 to 1997, *Salmonella* spp. was the pathogen most likely to be associated with consumption of fruits and vegetables at 48% (Sivapalasingam et al., 2004). A variety of *Salmonella enterica*

serovars have been studied and it was found that different serovars differ in their mechanism of adhesion (Berger et al., 2010). Various *Salmonella* adhesion mechanisms involve the use of aggregated fimbriae (known as Tafi), cellulose synthesis, and the O antigen capsule (Barak et al., 2005, Barak et al., 2007). Even biofilm formation was shown to play a role with *Salmonella* strains showing strong biofilm production capability being better able to adhere and persist on intact lettuce leaves for 9 days compared to weak biofilm producing strains (Kroupitski et al., 2009). *E. coli* has also been shown to have multiple mechanisms for adhering to produce. The main leaf attachment mechanisms are by curli (Jeter and Matthysse, 2005), EspA filaments of the filamentous type III secretion system (Knutton, 1995), and also via flagella (Xicohtencatl-Cortes et al., 2009).

While the mechanisms for produce attachment have not been elucidated as well for *L. monocytogenes*, there are still connections to be made in how biofilm production could play a significant role in produce attachment since *L. monocytogenes* is capable of biofilm formation (Djordjevic et al., 2002). Biofilms establish on the surface of the attachment site as three-dimensional multicellular structures. Biofilm formation begins when cells on a surface interact with each other and the surface itself before initiating production of extracellular polymeric substances (EPS) such as polysaccharides and proteins. These EPS form the extracellular matrix upon which the cohesion of the biofilm is dependent (Branda et al., 2005). It is also possible that *L. monocytogenes* could incorporate into heterogenous biofilms that are already on the plant surface and formed by epiphytic bacteria which could enable a protected environment for the

pathogen (Aruscavage et al., 2006). While *L. monocytogenes* lacks any type of Tafi or curli, it does have flagella, which may contribute to plant colonization ability, and also allows for motility which has also been shown to positively affect colonization (Gorski et al., 2009). Using the polyvinyl chloride (PVC) microtiter plate biofilm assay, flagellar motility has also been shown to be essential in *L. monocytogenes* biofilm formation (Lemon et al., 2007). Additionally, recent studies have shown how certain proteins expressed by *L. monocytogenes* potentially aids in its attachment to produce. A serotype 4b strain was studied to elucidate which of 32 genes were upregulated when grown on lettuce leaves. Of these, a gene (*lcp*) containing a potential cellulose binding domain was significantly upregulated during growth on lettuce. *L. monocytogenes* mutants lacking *lcp* were found to have a significantly lower binding ability to lettuce compared to the wild type strain, suggesting that *Listeria* cellulose binding protein (LCP) may indeed have an essential role in produce attachment (Bae et al., 2013). Additionally, a Cnp/Fnr Family transcription factor named Lmo0753 was found to be especially prevalent in lineage II outbreak strains of *L. monocytogenes*. The transcription factor was also found to have similarities to the major virulence regulator PrfA in two of its functional domains. Again, mutants lacking *lmo0753* were found to have significantly lower attachment ability to romaine lettuce leaves than the wildtype or complement strains (Salazar et al., 2013). The above two studies show that there are indeed a variety of mechanisms, some of which may act in unison, available for *L. monocytogenes* to be able to attach to produce and allow its subsequent colonization.

The ability of a pathogen to grow once attached is also dependent on a variety of factors, some of which include availability of nutrients, availability of water and also the presence of competition among other microorganisms present in the phyllosphere (Aruscavage et al., 2006). As previously mentioned, bacterial colonization on a plant is unevenly distributed and bacterial aggregates are usually found where conditions are most favorable for growth. Hence, on leaves these aggregates are in between the crevices of epidermal cells, close to the stomata, along veins and at the base of trichomes (Leveau and Lindow, 2001). Otherwise healthy plants tend to naturally leach small amounts of minerals, sugars, and amino acids which supply plant microflora with carbon and nitrogen sources (Tukey, 1970). The main sugars that are leached are fructose, glucose and sucrose (Mercier and Lindow, 2000). Roots are also able to be colonized by certain bacteria as nutrients leach from root tips as well as from the areas of the root base from which the lateral roots form (Brandl, 2006). Tissue damage could also cause leaching of nutrients and allow for better conditions for microbial proliferation (Aruscavage et al., 2006). *L. monocytogenes* has been shown to be able to use the phosphotransferase system to preferentially uptake sugars, like glucose and fructose, and its ability to utilize a variety of other carbohydrates has been demonstrated. The pathogen, however, lacks the genes for synthesis of nitrate reductases and so requires reduced nitrogen compounds such as ammonium or nitrogen containing amino acids, such as glutamine, as a nitrogen source. Additionally, the ability to catabolize amino acids is unlikely since the genomic information does not show the respective pathways (Joseph and Goebel, 2007). While there have been

efforts to study nutrient metabolism in relation to the mammalian host cell cytosol, there is a dearth of information regarding *L. monocytogenes* nutrient metabolism in plants. Further studies regarding this topic need to be conducted. The distributions of plant microflora and any pathogens that may also grow could be dependent on the wettability of the colonizable tissue and hence, the amount of water present for use by the bacteria (Bunster et al., 1989). Additionally, higher relative humidity has been shown to better support the growth of bacterial communities and its continued colonization of the plant tissue (Cooley et al., 2003, Leben, 1988).

Once growth conditions are found to be appropriate for survival, pathogens are able to persist and sometimes even internalize in a plant. Both persistence and internalization will be discussed in later chapters since they pertain more to the experimental objectives. The results from studies already conducted, however, indicate that bacterial type and strain, produce type or even the interactions between the two are highly variable depending on the combination used.

1.3 Romaine lettuce

1.3.1 Lettuce background and production statistics

Lettuce, *Lactuca sativa*, is an annual, flowering plant of which seven main varieties exist. Varieties include crisphead, butterhead, loose leaf, celtuce (used for its stem), latin, oilseed (seeds pressed for oil) and romaine lettuce. Lettuce grows best in

fertile soil that is well-drained of excess water. In general, a germinating lettuce seed requires temperatures of between 35°F and 90°F but low temperature extremes can result in lack of germination in some varieties, while high temperature extremes can result in bolting in others (Organic Seed Alliance, 2010). Romaine lettuce specifically is a variety that forms long, upright leaves with thick ribs that run down the center and result in relatively looser heads. Its leaves are generally crisp and range from bright green to dark green in color for outer leaves. The inner leaves of Romaine lettuce which are sweeter and range from yellowish to light green are typically sold in the market as Romaine hearts (UC Davis, 2001). While lettuce varieties in general have been widely used in salads, Romaine lettuce gained much of its popularity in the United States via the introduction of the Caesar salad in which it features as the sole vegetable. It has also been used in Mediterranean cuisine for a long time (USDA ERS, 2005).

According to the United States Department of Agriculture Economic Research Service (USDA ERS), Romaine lettuce is one of the fastest growing crops in the United States in terms of production, export and consumption (USDA ERS, 2005). The variety of choices available to a consumer include commodity romaine, prepackaged romaine hearts, romaine in salad mixes or kits, and even romaine ubiquitously sold at salad bars or restaurants.

Domestic production of lettuce is mostly concentrated in California with 73% and Arizona with 22% of market share and only 5% of production being in other states such as Oregon, New York and New Jersey. In terms of world rankings, China is the only country ahead of the U.S. in lettuce production (USDA ERS, 2005). In line with its rising

popularity over the past few decades, the harvested acreage allotted to romaine lettuce production has increased from around 21,000 acres in 1992 to roughly 91,000 acres in 2013. Consequently, domestic production increased from 5,652 to 26,620 (in 1,000 cwt pounds) and value of production went from $99 million to $880 million in the same span of time. While head lettuce still accounts for a majority of per capita head lettuce consumption, the margin by which it exceeds romaine and leaf lettuce combined has dropped through the years. For example, in 1985, per capita consumption of all lettuce was 27.0 pounds (lbs) with head lettuce accounting for 23.7 lbs (88%) and romaine and leaf (reported as a combined unit) accounting for 3.3 lbs (12%). As of 2013, total lettuce per capita consumption was slightly down at 23.8 lbs but according to the USDA ERS breakdown, head lettuce only accounted for 12.5 lbs (53%) while romaine and leaf lettuce accounted for 11.3 lbs (47%) (USDA ERS, 2014).

1.3.2 Pathogens associated with lettuce and outbreaks

Between 1973 and 1997, the proportion of foodborne outbreaks attributed to produce increased from 0.7% in the 1970s to 6% in the 1990s. The main produce items implicated in that period were melons, seed sprouts, fruit juice, salads, and lettuce (Sivapalasingam et al., 2004). Between the 1990s and 2005, the proportion of outbreaks attributed to produce contamination increased even further to 13%. Most of the produce vehicles of contamination, however, remained the same (DeWaal and Bhuiya, 2009). Lettuce has noticeably been on the list of the most commonly implicated produce

items for quite a long period of time. A variety of pathogens have been isolated from lettuce including *Campylobacter* (Park and Sanders, 1991), *Salmonella* (Ercolani, 1976), *Staphylococcus* (Abdelnoor et al., 1983), *Aeromonas* (Callister and Agger, 1989), *E. coli* (Brandl, 2006), *Shigella* (FDA, 2003a) and *L. monocytogenes* (Tang et al., 1994).

Salmonella and *E. coli* O157:H7 are the two pathogens most commonly associated with produce (Rangel et al., 2005, Sivapalasingam et al., 2004). However, when it comes to lettuce specifically, if excluding viruses, *E. coli* is the next most predominant pathogen in causing outbreaks (DeWaal and Bhuiya, 2009). According to the FDA, there have been 18 outbreaks as a result of *E. coli* O157:H7 contamination in which fresh or fresh-cut lettuce has been implicated from the period of 1995 to 2005 (FDA, 2005). In fact, in the last 10 years since 2005 there have been 4 major outbreaks concerning the combination of *E. coli* and lettuce with a few less severe outbreaks scattered between them. In 2006, there was a multistate outbreak of *E. coli* O157:H7 involving foods consumed at Taco Bell. A total of 71 people in 5 different states fell ill with 53 hospitalizations and 8 cases developing hemolytic-uremic syndrome (HUS). Evaluation of epidemiological data indicated that the shredded lettuce used in a variety of menu items was the most likely source of the outbreak (CDC, 2006). Another outbreak involving *E. coli* O157:H7 and bagged, fresh-cut lettuce occurred during the same period in 2006 and caused 81 cases with 26 hospitalizations and 2 cases of HUS. Strains isolated from environmental samples taken from dairy farms located close to the area where the lettuce was grown was found to be a match to the outbreak strain (Doyle, 2008). Another 2 outbreaks occurred with one in 2008 affecting 134 people in

the U.S. and Canada and the other in 2010 affecting 26 people in the U.S. alone (Olaimat and Holley, 2012). The latest outbreak involving lettuce was one in December 2011 with romaine lettuce sold at a single grocery store chain being implicated. The outbreak spanned 60 cases in 10 states with 30 hospitalizations and 2 developing HUS (CDC, 2011). From these outbreaks, it is clear that *E. coli* has some advantage in colonization and persistence in lettuce.

As discussed earlier, there have been multiple produce outbreaks linked to *L. monocytogenes* contamination. However, there have been no outbreaks of lettuce linked to *L. monocytogenes* as of yet. According to Hanning et al., there are several reasons that may contribute to the lack of listeriosis cases from contaminated lettuce. Reasons include the short shelf life of lettuce, possible inhibition of *L. monocytogenes* growth due to lettuce background microflora, and the possibility that lettuce itself may have properties that prevent the persistence of *L. monocytogenes*. The authors, however, stress that these potential reasons need to be studied further and that the list is by no means exhaustive (Hanning et al., 2008). However, this does not mean that *L. monocytogenes* outbreaks involving romaine lettuce does not have the potential to happen given the right circumstances. Between the period of January 2010 and January of 2015, there were 87 recalls involving *L. monocytogenes* contamination in produce. Of these, 18 (~20%) were specific to romaine lettuce or romaine lettuce containing products (FDA, 2015). The disparity between the number of recalls and lack of outbreaks pertaining to *L. monocytogenes* and romaine lettuce is confounding. All samples tested for presence of *L. monocytogenes* undergo a selective enrichment step which serves to

both increase the pathogen's numbers and revive stressed cells. Perhaps the positive samples tested in recalls have too low of an initial count to cause notable cases of illness or if they do, it goes unreported or is misdiagnosed. It is also possible that lettuce is contributing to the number of sporadic cases instead of towards outbreaks themselves. While no lettuce outbreaks concerning *L. monocytogenes* have occurred yet, there is definitely potential for it to happen. An improper storage event, such as temperature abuse, at the production level could be sufficient to cause even low counts of *L. monocytogenes* to proliferate on lettuce and act as a vehicle of transmission to consumers.

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CHAPTER 2. PERSISTENCE OF *LISTERIA MONOCYTOGENES* IN ROMAINE LETTUCE

2.1 Abstract

Persistence studies are useful in that they provide an assessment of the food safety risks posed by human pathogens on produce. Persistence of 3 *Listeria monocytogenes* strains on 3 romaine lettuce cultivars was studied independently by inoculating seeds in 25 ml of 8 log CFU/ml for 30 minutes. Seeds were grown on various soil types (e.g. standard potting mix, Indiana top soil) or sterile soft-top agar for up to 60 days. Average CFU/g of *L. monocytogenes* retained on seeds or persisting on growing plants was calculated from a total of 5 replicates per harvest day. Results showed that plants grown on sterile soft-top agar maintained between 4.4 to 7.8 log CFU/g *L. monocytogenes* after a 60 day period, while pathogen levels dropped below the limit of detection (2 log CFU/g) by Day 18 on 75% Indiana top soil, and by Day 45 on commercial potting mix. These findings suggest that the higher the incidence of competitive soil microflora or the lower the surrounding relative humidity, the less likely *L. monocytogenes* is able to persist. Additionally, no significant differences in persistence were found between the 3 strains of *L. monocytogenes* used, each of which represented a different serotype and lineage.

Cultivar differences showed the potential to influence pathogen persistence but could not be confirmed. The presence of a clay coating on the seed (used by the industry to create a uniform size for mechanized planting) was not found to retain *L. monocytogenes* any differently than on the seed itself. These results indicate that romaine lettuce can support the growth of *L. monocytogenes,* potentially even up to the harvest period if ideal conditions are met. This emphasizes that prevention of pathogen contamination is as important at the pre-harvest stage as during post-harvest processing.

2.2 Introduction

The persistence of pathogens in produce has been studied under a variety of conditions and contamination routes (Dong et al., 2003, Islam et al., 2004b, Jablasone et al., 2005, Kisluk and Yaron, 2012). Understanding the ability of pathogenic bacteria to grow and remain on or in plant tissue can aid with pre-harvest control strategies, as well as can indicate the degree to which post-harvest sanitation methods must be successful in order to prevent outbreaks from consumption of contaminated produce.

There are a variety of sources present in a produce-growing environment that can contribute to contamination of the product. Various studies have simulated bacterial contamination by inoculation of the plant at different stages of growth with varying results. For example, a 2004 study used young lettuce and parsley

seedlings grown in open fields to study persistence of *E. coli* O157:H7 via contamination through compost amended manure inoculated with 10^7 CFU/ml of the pathogen or via contaminated irrigation water inoculated with 10^5 CFU/ml of the pathogen. *E. coli* O157:H7 was found to persist between 154 and 217 days in amended soil, but more concerning is the fact that *E. coli* was found on lettuce and parsley plants up to 77 and 177 days respectively past the contamination event as a seedling (Islam et al., 2004a). Using similar methods but with an avirulent strain of *Salmonella enterica* serovar Typhimurium, the same authors found that the pathogen was detectable on lettuce for 63 days and on parsley for 231 days respectively. Additionally, *Salmonella* persisted in the contaminated compost amended soil for between 161 and 231 days (Islam et al., 2004d). *Salmonella enterica* serovar Typhimurium was used in another similar study, but this time using carrots and radishes. Post seed-sowing in contaminated manure, *Salmonella* was found on carrots up to 203 days and on radishes up to 84 days (Islam et al., 2004c). The above studies show that not only are pathogens able to persist for a commercially viable amount of time on those particular produce types mentioned, but that there are differences in the persistence of the pathogen depending on the produce type itself. Since plant type and bacterial strain combinations have been shown to vary (Dong et al., 2003, Golberg et al., 2011), as many combinations as possible need to be studied in order to avoid making generalizations about produce-pathogen relationships. With additional influential factors such as varying climate

conditions and contaminant levels, true risk assessment may be hard to achieve but is still very much necessary for the safety of consumers.

When unable to study persistence in a field setting, researchers often turn to the use of greenhouses or growth chambers. This allows for control of conditions such as humidity, temperature, and even light cycles. While not fully emulating what occurs in traditional produce cultivation in a field, it allows for a simulation of it while being better able to define research needs. Moreover, it reduces the presence of confounding factors, such as unpredictable weather changes or roaming animals, that may otherwise be found in a field setting and have the potential to influence results. Kisluk et al. (2012) investigated the persistence of *Salmonella enterica* serovar Typhimurium on parsley grown in a greenhouse setting. Persistence was tracked on the phyllosphere and rhizosphere individually following spray irrigation with contaminated water at 8.5 log CFU/ml. Only an hour past the irrigation challenge, it was found that the phyllosphere retained the highest initial levels of *S*. Typhimurium followed by soil levels and then rhizosphere levels. This trend continued for the rest of the experimental period. After 28 days, *Salmonella* was recovered from the phyllosphere, with the leaves and stalks resulting in 3.9 and 3.4 log CFU/g respectively. *Salmonella* was also recovered from the rhizosphere at 2.0 log CFU/g, while soil levels were found to be 2.2 log CFU/g (Kisluk and Yaron, 2012). Using growth chambers, Cooley et al. studied the persistence of *Salmonella enterica* serovar Newport and *E. coli* O157:H7 on *Arabidopsis thaliana*. When roots and shoots were monitored for growth, it was found that the roots had almost 30 to 50

times the number of pathogens as present on the leaf tissue. The authors hypothesized that the reduced availability of nutrients in the plant foliage over time could not support the growth of as many pathogens (Cooley et al., 2003). These experiments demonstrate that pathogen distribution and persistence on a plant is not evenly distributed. Depending on the type of plant and the method of contamination, significant numbers of pathogenic bacteria may be able to persist on the phyllosphere or the rhizosphere of the plant. Since either of these portions, and sometimes even both, are edible depending on the type of produce, this constitutes a health risk if consumed.

Other factors such as interaction of plant microflora, or even the plant with the pathogen itself, can affect the persistence of a pathogen. Interactions of the pathogen with the plant microflora may either help boost its persistence or reduce its numbers depending on the conditions created. Growth of a pathogen can be positively influenced if plant microflora or other plant pathogens aid in degrading plant compounds into more easily metabolized carbon sources for the pathogen. Certain plant pathogens may have enzymes that are better able to process plant material than human pathogens such as pectolytic enzymes, cellulases, and cutinases which all degrade some of the most abundant and complex plant substrates (Agrios, 2005). Additionally, plant microflora can influence a pathogen's ability to survive by damaging the plant tissue and causing leakage of nutrients or by changing other aspects of the microenvironment. When co-inoculated with soft rot bacteria, *S.* Typhimurium had a 1 log higher population size on carrots, peppers and

potatoes than when inoculated alone (Beuchat, 2002). Another study showed *E. coli* O157:H7 ability to increase its population size by 4.0, 4.5, and 11 fold on lettuce leaves that had been mechanically bruised, cut into large pieces, and shredded respectively. In comparison, leaves that were left intact only had a 2 fold increase in population size (Brandl, 2008). Some microflora can also inhibit pathogen growth as shown by the inhibition of *L. monocytogenes* growth on potato tuber slices by *Pseudomonas fluorescens* and *Pseudomonas viridiflava* (Liao and Sapers, 1999). With regards to changes in the microenvironment, one study showed that in apples decayed by *Glomerella cingulata*, *L. monocytogenes* was able to proliferate better because the pH of the apple tissue was increased to 7.0 by the fungus. However, when the apple was decayed by *Penicillium expansum, L. monocytogenes* populations were not detectable after 5 days due to the pH of the tissue dropping to 3.7 (Conway et al., 2000). This shows again that pathogen proliferation is influenced by many factors and growth of a pathogen can be negatively or positively affected depending on the specific microflora present in the vicinity of its colonization. Plant defenses may also help prevent persistence of a pathogen depending on the success of the plant innate immune response to pathogen associated molecular patterns or effector triggered immunity (Deering et al., 2012).

While there are a relative abundance of pre-harvest studies with *E. coli* O157:H7 and *Salmonella enterica*, there are few studies regarding pre-harvest persistence of *L. monocytogenes* in produce. Of the *Listeria* persistence studies on lettuce currently found in literature, most focus on post-harvest persistence of the

pathogen under various conditions (Koseki and Isobe, 2005, Oliveira et al., 2010, Takeuchi et al., 2000). The understanding of plant and pathogen interaction throughout all stages of production, including pre-harvest stages, is important in being able to mitigate pathogen presence. The objective of this study was to determine the persistence of *L. monocytogenes* on romaine lettuce using various combinations of pathogen strains, lettuce cultivars, as well various types of growth medium in order to better understand their interactions.

2.3 Materials and Methods

2.3.1 Bacterial strains and storage

Listeria monocytogenes strains 10403S, FSL B2-107, FSL J1-194, and FSL J1-208 were used in this study. All bacterial strains were stored at -80°C in Brain-Heart Infusion (BHI; Bacto™, Sparks, MD) broth supplemented with 25% glycerol. Prior to the start of each plant growth curve, stock cultures were streaked onto BHI agar plates to obtain isolated colonies. A single colony was used to inoculate each of 6 tubes containing 5 ml of sterile BHI broth per tube. Cultures were incubated overnight (16-18 hours) at 35°C with shaking at 200 rpm.

2.3.2 Plant germination rates

Germination rates of Dole Fresh Vegetables, Inc. (Westlake Village, CA) top three romaine lettuce cultivars were established by growing them in a petri dish.

Two layers of paper towel were placed in an inverted petri dish and 5 ml of water was added to moisten it. Ten seeds per cultivar were placed in each petri dish and this was done in duplicate. Plates were closed, sealed with parafilm and left in the dark at ambient temperature for 3 days. The number of germinated seeds out of the 10 in a petri dish were counted and expressed as a percentage.

2.3.3 Background microflora of non-inoculated seeds

Levels of background microflora on non-inoculated seeds were observed by plating on Plate Count Agar (PCA; Difco™, Sparks, MD). A total of 5 seeds from each cultivar were each placed into an Eppendorf tube containing 200µl of 0.1 M phosphate buffer pH 7.0 and left to soak overnight at room temperature to allow the clay coating to soften. A sterile disposable mini pestle was used to grind each seed within the Eppendorf tube until a homogenized suspension was achieved. A 100 µl volume was plated directly onto PCA for a dilution of 10^{-1} while the rest of the seed suspension was used to make dilutions of up to 10^{-5} using phosphate buffer as the diluent. All dilutions were plated on PCA and the plates incubated for 2 days at 30°C.

Non-inoculated seeds were also plated on Modified Oxford Agar (MOX; Difco™, Sparks, MD) to check for presence of any background *Listeria* species. A total of 5 seeds from each of the three cultivars were individually ground in a mortar and pestle with 10ml of 0.1 M phosphate buffer, pH 7.0. Dilutions of 10^{-2} to 10^{-5} of each

of the ground seed suspensions were plated on MOX and incubated for 2 days at 30°C.

2.3.4 Plant growth curves

Overnight cultures of the particular strain of *Listeria monocytogenes* (refer to Table 1 for specifics of each trial) were grown in BHI at 35°C with shaking at 200 rpm. The culture was washed to remove the growth medium by centrifuging the culture at 3000 rpm for 6 minutes and resuspending the cell pellet in 0.1 M phosphate buffer pH 7.0 by vortexing (repeated 3X). Enumeration of the washed bacterial culture was conducted by plating dilutions on MOX and incubated at 30°C for 2 days. Roughly 80 seeds from each cultivar were soaked in the washed culture and rotated using a Labquake® shaker (Labindustries, Inc., Berkeley, CA) for 30 minutes at room temperature. Seeds were then drained from the culture and left to air dry on filter paper in a biosafety cabinet for 1 hour. After drying, 5 seeds were harvested by grinding each one with a mortar and pestle with 10ml of 0.1 M phosphate buffer pH 7.0. Enumeration of initial seed contamination levels was done by spread plating dilutions of the homogenized mixture onto MOX and incubating the plates for 2 days at 30°C. Remaining air dried seeds were individually and sterilely placed into 25x200mm sterile test tubes containing 20 ml of 0.8% soft top agar (Bacto™,Sparks ,MD) as the growth substrate. The tubes were sealed with parafilm before being placed in a BioSafety Level 2 greenhouse maintained at an average temperature of 28°C with a 16/8 hour light-dark cycle.

Growth curve trials were also conducted in varying types of soil or potting mix. The soil was prepared by mixing 2.5 gallons of either Professional grower's potting mix (Sun Gro Sunshine Redi-Earth, Bellevue, WA) or 75% Indiana top soil mixed with 25% potting mix (Sun Gro Metro-mix 510, Bellevue, WA) with 1.5 tablespoons of Marathon® 1% Granular (OHP, Inc., Mainland, PA) as an insecticide. This was aliquoted into planter trays. Seeds inoculated as described above were planted approximately 1 inch below the soil surface. Planter trays were placed into larger holding trays with water and slow-release fertilizer, Osmocote® (Scotts Miracle-Gro, Marysville, OH) added.

Harvesting was conducted every 3 days for up to 21 days followed by an additional 3 total extension harvests up to 60 days post seed inoculation. At each harvest, 5 seeds were sampled as described for the initial seed contamination and average CFU/plant and CFU/gram of plant was obtained. Plants that grew too big to fit in the mortar were ground in a sterilized blender using 100 ml of 0.1 M phosphate buffer pH 7.0 instead of 10ml, thereby reducing the limit of detection to 10^3.

Controls for each growth curve were in the form of non-inoculated seeds which were rotated in 0.1 M phosphate buffer pH 7.0 instead of culture. Planting and placement in the greenhouse were identical to the procedure stated above. Only one untreated seed was harvested at a time, and at random to ensure that no *Listeria monocytogenes* colonies were observed.

Results from the plant growth curves were analyzed via 2-factor ANOVA ($P < 0.0001$) using JMP 11 statistical software (SAS Institute Inc., Cary, NC) and individual

differences were tested using Tukey's honest significant difference (Tukey's HSD) test ($\alpha = 0.05$). Treatment, day, and the interactions of the two were used as predictive variables with log CFU/g as the response.

2.3.5 Determining the presence of *Listeria monocytogenes* in potting mix or soil used for non-test tube trials

Both *Listeria* enriched and non-enriched samples of potting mix (Sun Gro Sunshine Redi-Earth) and 75% Indiana top soil were used to check for any background levels of the pathogen. Non-enriched samples were tested by stomaching 25 grams of planting medium with 225 ml of 0.1 M phosphate buffer pH 7.0, using a Stomacher® 400 Circulator (Seward, Davie, FL), for 1 minute at 280 rpm. Dilutions of 10^{-2} to 10^{-6} were spread plated onto MOX plates and incubated at 30°C for 2 days. General background microflora of non-enriched samples was also enumerated. Dilutions of 10^{-2} to 10^{-6} were spread plated onto PCA and incubated at 30°C.

Planting medium enriched for *Listeria* were tested by stomaching 25 grams of the planting medium with 225 ml of Buffered Listeria Enrichment Broth (BLEB; Difco™, Sparks,MD) for 1 minute at 280 rpm. The homogenate was incubated for 4 hours at 30°C. This was followed by adding 900µl of *Listeria* Selective Enrichment Supplement (LSES; 3.6 mg/ml sodium hydroxide; 9mg/ml nalidixic acid; 2.25 mg/ml acriflavine; 11.25 mg/ml cycloheximide; in 10% methanol aqueous solution) and

further incubation at 30°C for an additional 44 hours. Dilutions of 10^{-2} to 10^{-6} were spread plated onto MOX plates and incubated at 30°C.

Any esculin positive colonies that grew on MOX plates were streaked onto *Listeria monocytogenes* chromogenic plating medium *(*LMPM; R&F Laboratories, Downers Grove, IL) to determine whether the colony was *L. monocytogenes.* LMPM *plates were incubated at* 30°C for 2 days.

Table 1: Summary of the *L. monocytogenes* strains, cultivars, and growth media tested to examine growth and persistence of the bacterium on various cultivars of lettuce

Trial	***Listeria monocytogenes* strain**	**Cultivar**	**Conditions**
T1	10403S	Braveheart	Sterile 25x200mm test tube with 0.8% Bacto agar. Parafilm sealed.
T2	10403S	Sun Valley	Sterile 25x200mm test tube with 0.8% Bacto agar. Parafilm sealed.
T3	10403S	Sun Belt	Sterile 25x200mm test tube with 0.8% Bacto agar. Parafilm sealed.
T4	FSL J1-194	Braveheart	Sterile 25x200mm test tube with 0.8% Bacto agar. Parafilm sealed.
T5	FSL J1-194	Sun Valley	Sterile 25x200mm test tube with 0.8% Bacto agar. Parafilm sealed.

Table 1 continued

T6	FSL J1-194	Sun Belt	Sterile 25x200mm test tube with 0.8% Bacto agar. Parafilm sealed.
T7	FSL J1-208	Braveheart	Sterile 25x200mm test tube with 0.8% Bacto agar. Parafilm sealed.
T8	10403S	Braveheart (no clay coating)	Sterile 25x200mm test tube with 0.8% Bacto agar. Parafilm sealed.
T9	10403S	Braveheart	Professional grower's potting mix (Sun Gro Sunshine Redi-Earth, Bellevue, WA).
T10	10403S	Braveheart	Autoclaved professional grower's potting mix (Sun Gro Sunshine Redi-Earth, Bellevue, WA).
T11	10403S	Braveheart	75% Indiana top soil mixed with 25% potting mix (Sun Gro Metro-mix 510, Bellevue, WA).
Note: All trials were conducted in a BioSafety Level 2 greenhouse under greenhouse conditions of 16/8 hour light cycles and temperatures of between 26-30°C.			

2.4 Results

2.4.1 Plant germination rates

Seeds from Dole with germination rate values of 98%, 99% and 99% for Braveheart, Sun Valley and Sunbelt cultivars respectively were obtained (Dole Fresh Vegetables Inc., 2014). When conducted in the lab, germination rates were found to be 100% for Braveheart, 100% for Sun Valley and 95% for Sunbelt. Germination rates were sufficiently high to proceed with remainder of experiment.

2.4.2 Background microflora of non-inoculated seeds

A small variety of bacteria grew on a portion of the seeds from each cultivar that were plated on PCA. Microbial counts ranged from less than 15 colonies per seed on Sunbelt and Braveheart cultivars to a maximum of 10^4 colonies on certain Sun Valley seeds. Background microflora on seeds included small, yellow colonies, large white colonies and some mold (Figure 1). Seeds from each cultivar were also plated on MOX, but none of the plated seed homogenates resulted in growth on the plate. No *L. monocytogenes* or any other esculin positive colonies were detected.

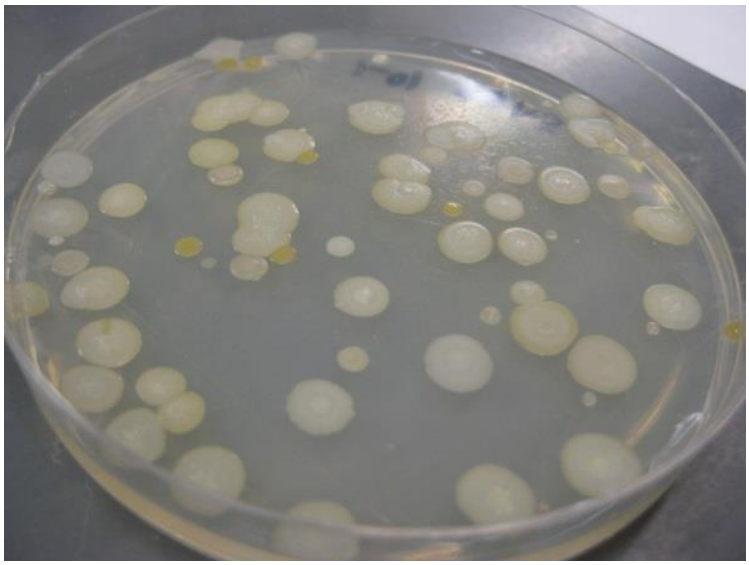

Figure 1: Example of the background microflora on Sun Valley seed homogenate plated on Plate Count Agar

2.4.3 Plant growth curves of *L. monocytogenes* persistence

As previously mentioned, romaine lettuce plants were grown from seeds that were exposed to a brief contamination event. Any MOX plates that did not show growth were listed as 2 log CFU/g as that was the limit of detection. Even though 25 ml of an initial concentration of approximately 10^8 CFU/ml was used for all trials, Day 0 was not taken into account since *L. monocytogenes* colonies enumerated from the seeds directly after the brief contamination event greatly varied between the different trials. Additionally, any data recorded beyond the first 21 day period was for observational purposes and was not included in statistical analysis since it was not part of the original experiment, and some observations were conducted on differing days. In addition, romaine lettuce plants grown in sterile tubes did not grow as well as plants in soil or potting mix trials.

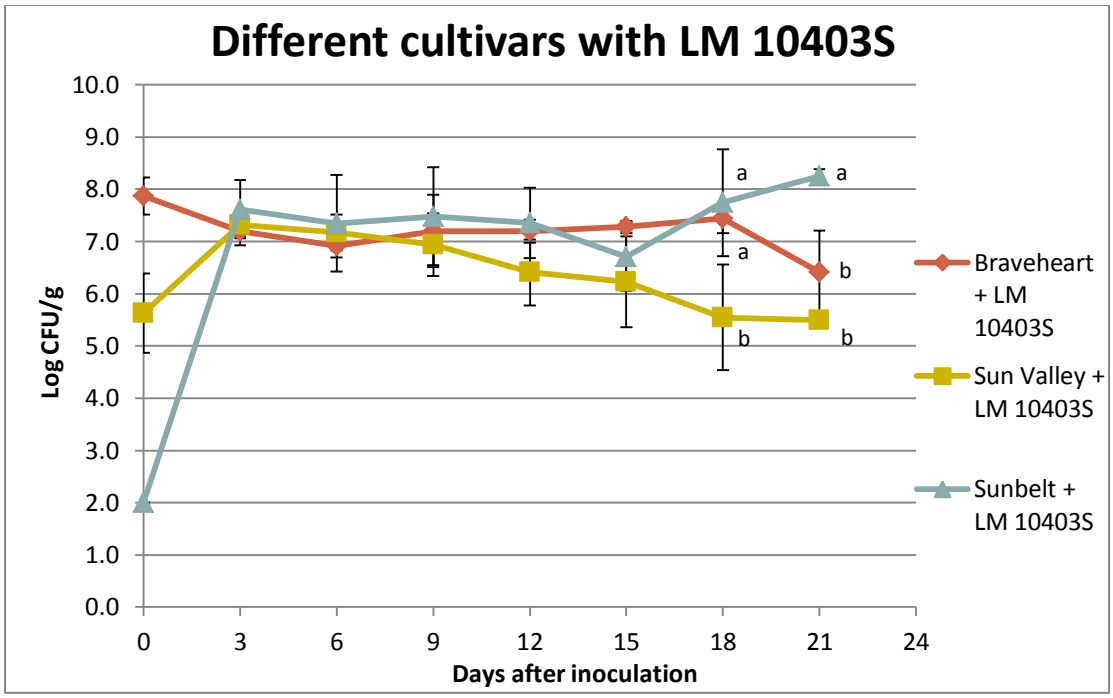

Figure 2: Effect of cultivar when used with same strain of *L. monocytogenes* 10403S for a 21 day period (grown in sterile test tubes). Data points for each day reported are based on the average CFU/g values of 5 replicates. Error bars denote standard deviations. Distinct letters represent statistically significant differences between data points for a specific day (Tukey's HSD; $p<0.05$).

Romaine lettuce seeds from 3 different cultivars (Braveheart, Sun Valley, and Sunbelt) were each treated with *L. monocytogenes* 10403S (Figure 2). Each of the cultivars started with differing *L. monocytogenes* concentrations on Day 0 with Braveheart, Sun Valley and Sunbelt having 7.87, 5.62, 2.00 log CFU/g respectively (Fig. 2). By Day 3, however, all cultivars had slightly above 7 log CFU/g. *L. monocytogenes* persistence on the Braveheart cultivar steadily increase until day 21 when there was a roughly a 1 log CFU/g decrease in *L. monocytogenes* enumerated

at Day 18 (Fig. 2). At the end of the 21 day period, a total of 6.41 log CFU/g of *L. monocytogenes* 10403S was enumerated from the Braveheart cultivar. After Day 3, there was a gradual decrease in the persistence of *L. monocytogenes* on the Sun Valley cultivar for a final total of 5.50 log CFU/g on Day 21. The Sunbelt cultivar, however, had an increase in persistence by approximately 1.50 log CFU/g between Day 15 and Day 21 to end at a high of 8.25 log CFU/g (Fig. 2). When statistical analysis was performed on the above trials, only a few of the days were found to be significantly different. Between Braveheart and Sun Valley cultivars, only Day 18 showed a significant difference. Between Braveheart and Sunbelt cultivars, significant differences were observed on Day 21 only. Between Sun Valley and Sunbelt, however, both Day 18 and Day 21 showed a significant difference in persistence. Therefore, statistically significant differences between cultivars were not observed for the first 15 days of growth (Fig. 2).

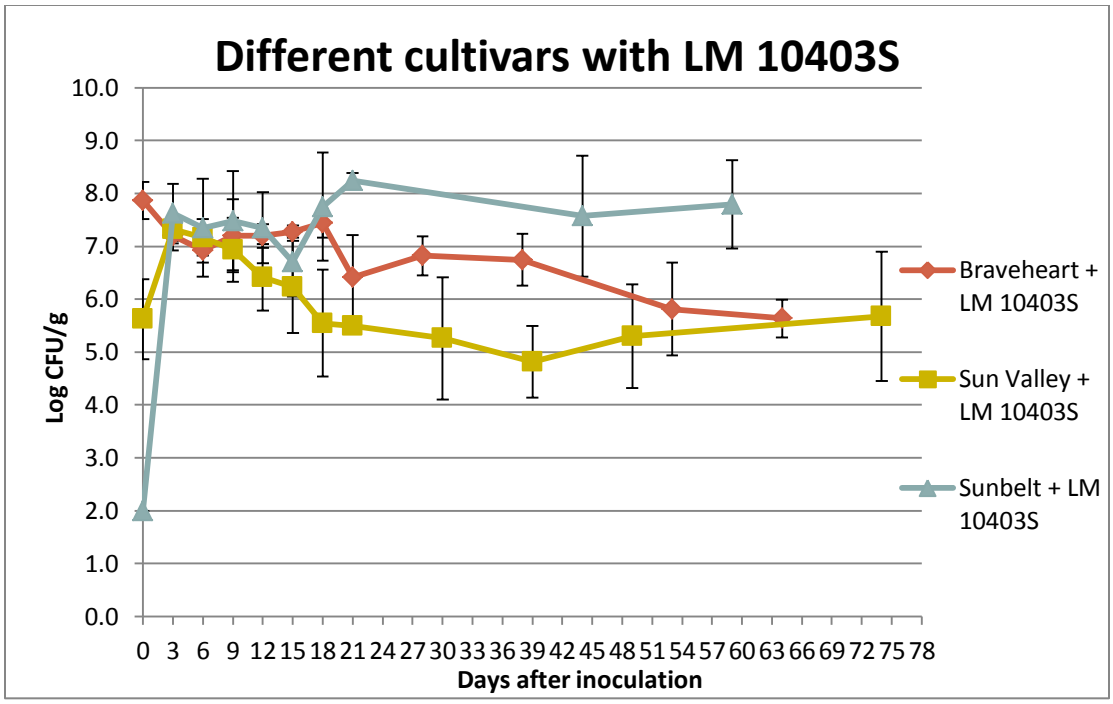

Figure 3: Effect of cultivar when used with same strain of *L. monocytogenes* 10403S throughout the trial (grown in sterile test tubes). Data points for each day reported are based on the average CFU/g values of 5 replicates. Error bars denote standard deviations.

When plants were sampled at extended times for observational purposes, differences in cultivars seemed to be more pronounced. However, statistical analysis could not be performed since some data collection points were on different days. Final data points collected were all conducted around or past the typical commercial harvest period of romaine lettuce. Based on the final data collection point for each trial, final *L. monocytogenes* concentrations on Braveheart, Sun Valley, and Sunbelt cultivars were 5.63, 5.67, and 7.79 log CFU/g respectively (Figure 3). The Sunbelt cultivar had levels of *L monocytogenes* enumerated from the plants that were

almost 2 log higher than the Braveheart cultivar (the lowest enumeration of *L. monocytogenes*). Thus, *L. monocytogenes* 10403S seems to persist better on the Sunbelt cultivar. Based on Figure 3 above, it is entirely possible that there are cultivar differences that are observed beyond Day 18 onwards. Regardless, comparisons of these graphs indicate that in ideal conditions, *L. monocytogenes* can persist to relatively high levels, even up to the harvest period of romaine lettuce.

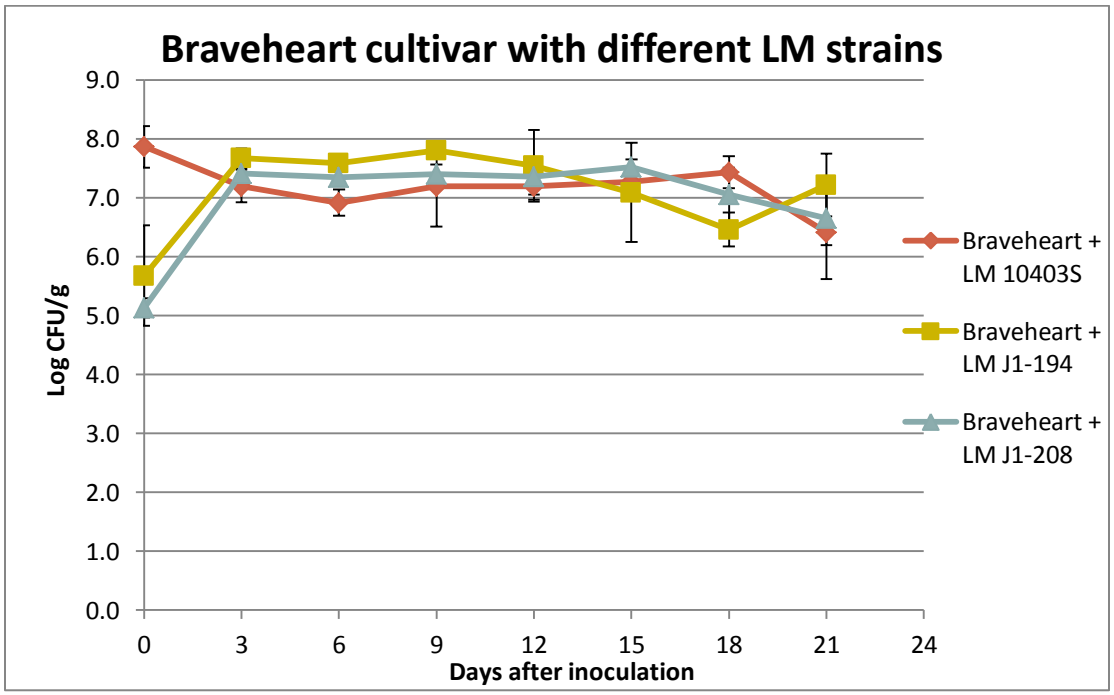

Figure 4: Effect of strain of *L. monocytogenes* when used with same Braveheart cultivar for a 21 day period (grown in sterile test tubes). Data points for each day reported are based on the average CFU/g values of 5 replicates. Error bars denote standard deviations.

Another set of experiments were conducted to determine how different isolates of *L. monocytogenes* grow on a particular cultivar (Figure 4). The cultivar used (Braveheart) is kept the same across trials while the strain of *L. monocytogenes* used is the variable factor. As with the other trials, there is a variation in enumeration on Day 0, however, all trials recover to similar values above 7 log CFU/g by Day 3. Trials testing persistence of different strains on the same cultivar seemed to follow a similar persistence pattern over the 21 day period tested. Final values of the different strains of LM 10403S, LM FSL J1-104, LM FSL J1-208 on Day 21 were 6.41, 7.22, and 6.65 log CFU/g respectively (Fig. 4). Based on the Tukey HSD statistical analysis, there were no significant differences between any of the days for any of the above 3 trials. Thus, for the 21 day period tested, strain differences were not significant.

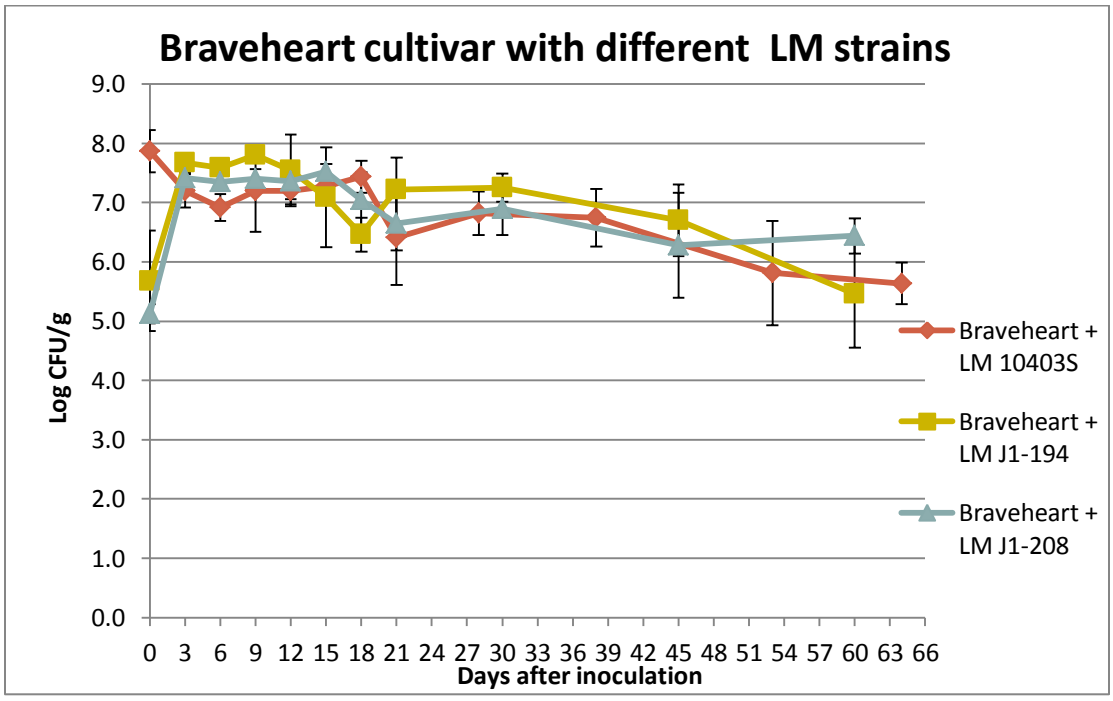

Figure 5: Effect of strain of *L. monocytogenes* when used with the same Braveheart cultivar throughout the trial (grown in sterile test tubes). Data points for each day reported are based on the average CFU/g values of 5 replicates. Error bars denote standard deviations.

For sample points beyond the 21 day period, persistence of all 3 strains tested continued to follow a similar pattern with only very slight variations in data. LM 10403S, LM FSL J1-104, LM FSL J1-208 on or beyond Day 60 were 5.63, 5.45, and 6.43 log CFU/g respectively (Figure 5). Based on the comparison graph above, it is unlikely that strain differences contribute to changes in persistence on a single cultivar.

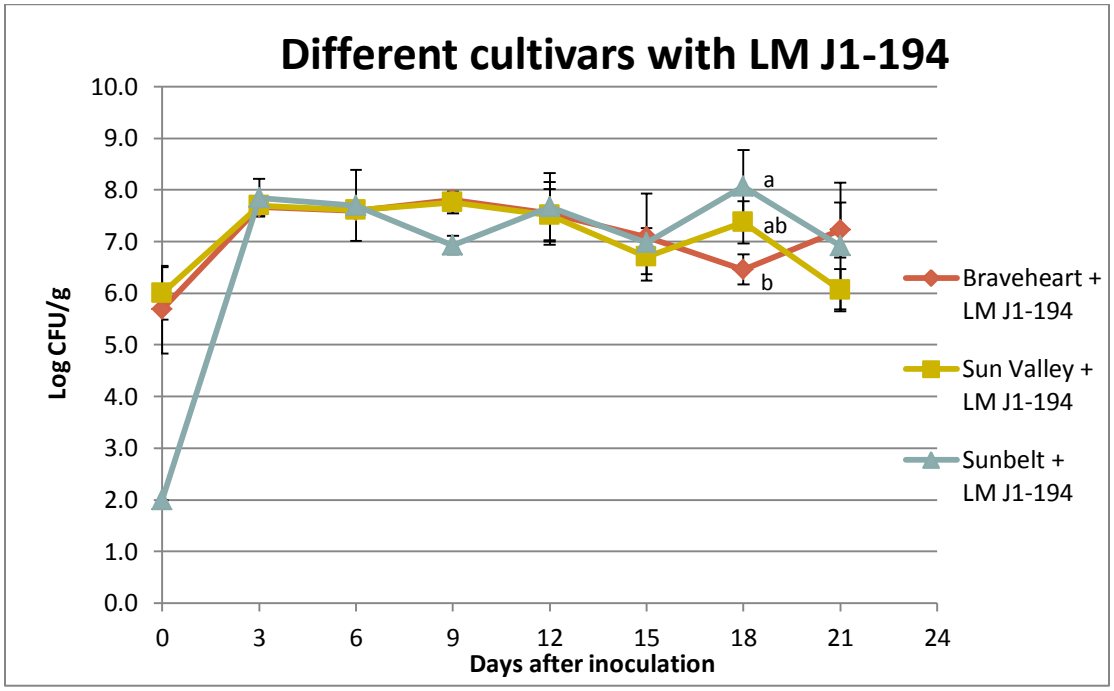

Figure 6: Effect of cultivar when used with same FSL J1-194 strain of *L. monocytogenes* for a 21 day period (grown in sterile test tubes). Data points for each day reported are based on the average CFU/g values of 5 replicates. Error bars denote standard deviations. Distinct letters represent statistically significant differences between data points for a specific day (Tukey's HSD; $p<0.05$).

A separate trial was conducted to test for the contribution of cultivar differences to *L. monocytogenes* persistence. The 3 cultivars (Braveheart, Sun Valley, and Sunbelt) were inoculated with *L. monocytogenes* FSL J1-194 as previously described. As shown in Figure 6, each of the cultivars started with differing levels of *L. monocytogenes* on Day 0 with Braveheart, Sun Valley and Sunbelt having 5.68, 5.99, 2.00 log CFU/g respectively. Again, by Day 3, all cultivars had the same levels of *L.* monocytogenes with slightly above 7.50 log CFU/g each. *L. monocytogenes* J1-194 persistence on the Braveheart cultivar increased until Day 9 before gradually decreasing at Day 18 and increasing to a final Day 21 value of 7.22 log CFU/g. Persistence of *L. monocytogenes* J1-194 on the Sun Valley cultivar decreased and then increased slightly on Day 18. The number of *L. monocytogenes* cells decreased again on Day 21 for a final concentration of 6.05 log CFU/g on Day 21. The Sunbelt cultivar, however, seemed to fluctuate throughout the 21 day period, ending with a final concentration of 6.59 log CFU/g. Statistical analysis of the trials listed in Fig. 6 was performed and the only data point which showed a significant difference in persistence was Day 18 between the Braveheart and Sunbelt trials (both treated

with FSL J1-194). Based on the above findings, cultivar differences are not significant for the 21 day period tested when using strain *L. monocytogenes* FSL J1-194.

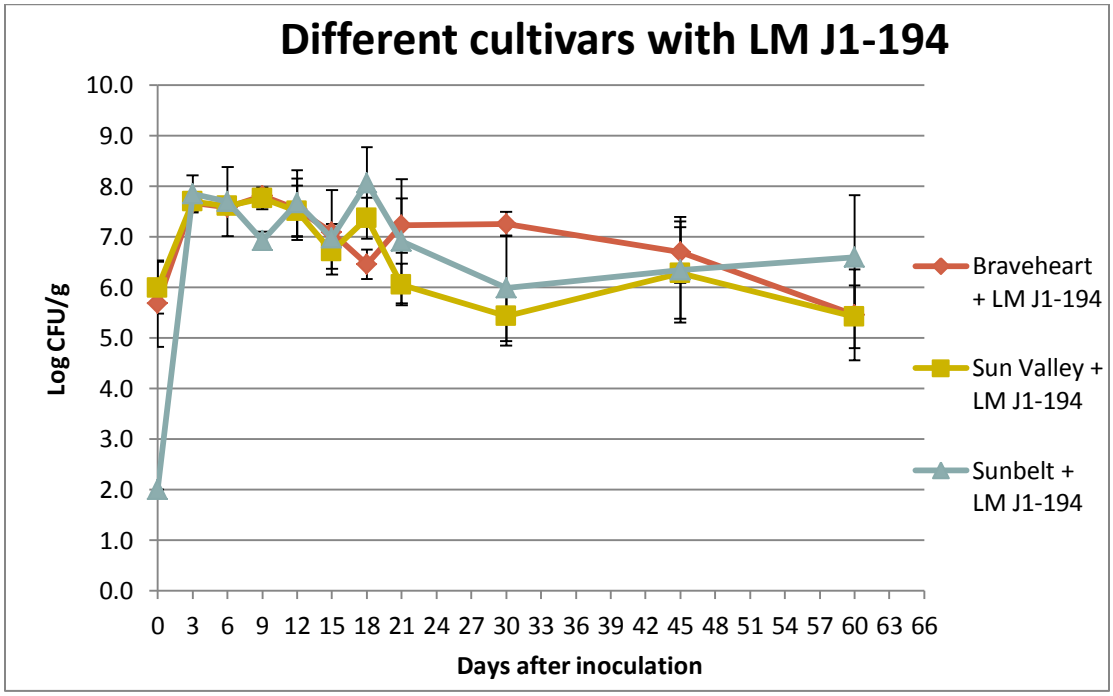

Figure 7: Effect of cultivar when used with same FSL J1-194 strain of *L. monocytogenes* throughout the trial (grown in sterile test tubes). Data points for each day reported are based on the average CFU/g values of 5 replicates. Error bars denote standard deviations.

When the sampling was extended to 60 days, the different cultivars showed slightly more variation in the persistence of *L. monocytogenes*, especially around Day 30 (Figure 7). There was an almost 2 log difference between the Braveheart and Sun Valley cultivars on Day 30 before the values converged again around Day 45. Final values for cultivars contaminated with *L. monocytogenes* FSL J1-194 after a

period of 60 days were 5.45, 5.41 and 6.59 log CFU/g for Braveheart, Sun Valley and Sunbelt respectively. Again, the Sunbelt cultivar showed noticeably higher (approximately 1 log CFU/g higher) persistence of *L. monocytogenes* at the end of the experimental period compared to the other 2 cultivars (Fig. 7).

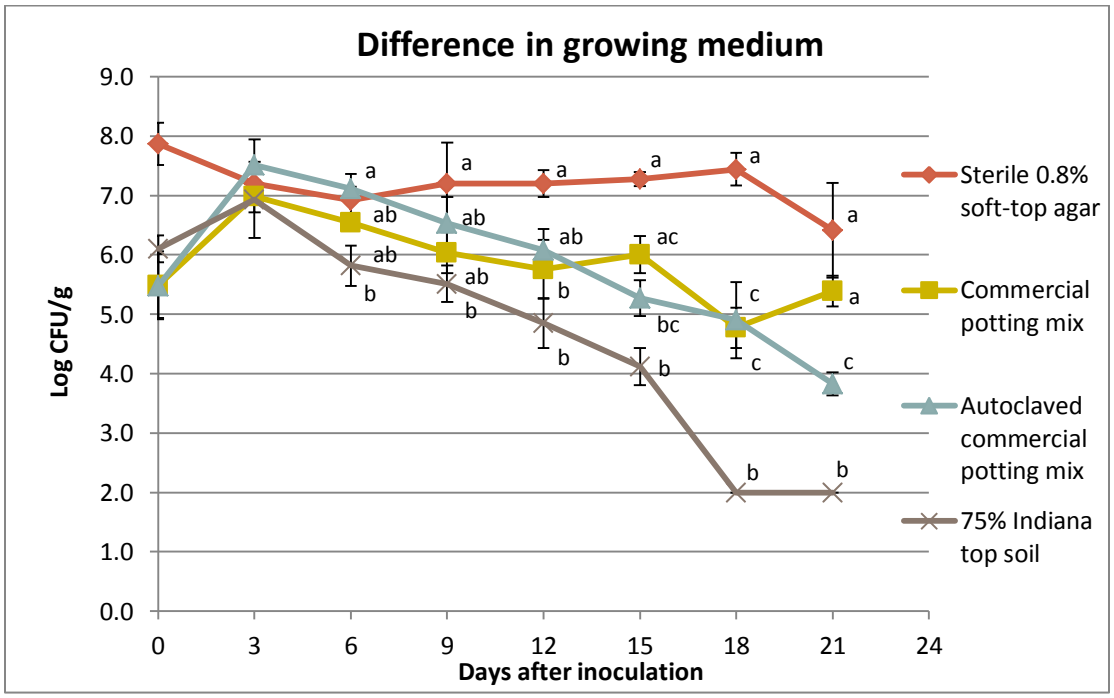

Figure 8: Effect of different types of growing media on *L. monocytogenes* 10403S persistence on Braveheart cultivar for a 21 day period. Data points for each day reported are based on the average CFU/g values of 5 replicates. Error bars denote standard deviations. Distinct letters represent statistically significant differences for a specific day (Tukey's HSD; $p<0.05$).

In order to test how persistence was affected by the medium that the romaine lettuce seeds were grown in, Braveheart cultivar seeds were inoculated

with *L. monocytogenes* 10403S and planted in various ways. As shown in Figure 8, contaminated seeds grown in sterile soft-top agar had the highest persistence after a period of 21 days followed by commercial potting mix (Sun Gro Sunshine Redi-Earth), autoclaved commercial potting mix and 75% Indiana top soil at 6.41, 5.39, 3.82 and 2.00 log CFU/g respectively. *L. monocytogenes* concentrations on 75% Indiana top soil had actually decreased to below the limit of detection by Day 18. It was found that commercial potting mix had relatively lower levels of microbial background when plated. Persistence on commercial potting mix compared to autoclaved commercial potting mix was not found to differ at a significant value other than for Day 21. When grown in sterile test tubes, *L. monocytogenes* concentrations were found to differ significantly from commercial potting mix on Days 12 and 18 only, from autoclaved potting mix on Days 15, 18 and 21, and from 75% Indiana top soil on Days 9 through 21. Both commercial potting mix and autoclaved potting mix trials differed significantly from the 75% Indiana top soil trial on Days 15, 18 and 21 (Fig. 8). The overall trend shows that persistence on plants grown in sterile test tubes differed quite largely from those grown in 75% Indiana top soil while commercial and autoclaved potting mix followed a similar trajectory down the middle. Hence, it can be concluded that *L. monocytogenes* is far more persistent in a closed, sterile environment than in an open, competitive environment. Persistence of approximately 4-5 log CFU/g is still possible in an open but relatively sterile environment (such as commercial potting mix) for a period of up to 21 days.

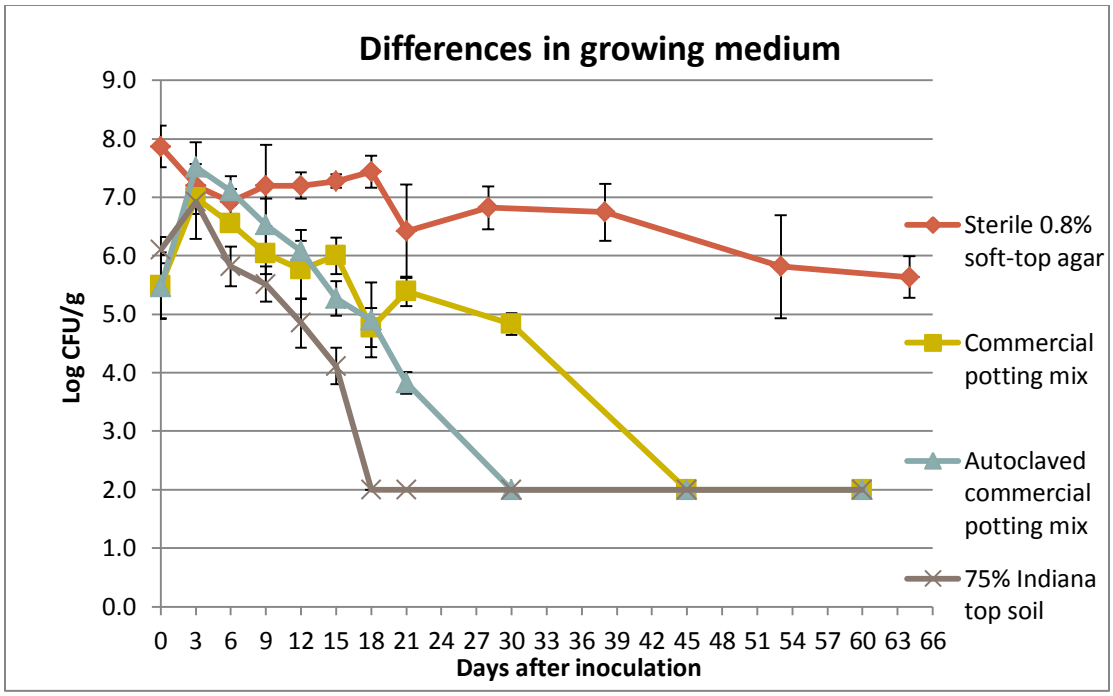

Figure 9: Effect of different types of growing media on *L. monocytogenes* 10403S persistence on Braveheart cultivar throughout the trial. Data points for each day reported are based on the average CFU/g values of 5 replicates. Error bars denote standard deviations.

Differences in the growth medium trials were more pronounced once extended to the 60 day trials (Figure 9). *L. monocytogenes* concentrations were below the limit of detection by Day 18 on 75% Indiana top soil, by Day 30 on autoclaved commercial potting mix, and by Day 45 on commercial potting mix. Only contaminated seeds grown in sterile soft-top agar were able to persist until the typical harvest period for romaine lettuce. Contaminated seeds grown in any of the

soil or potting mix types did not persist until the harvest period of approximately 60 days (Fig. 9).

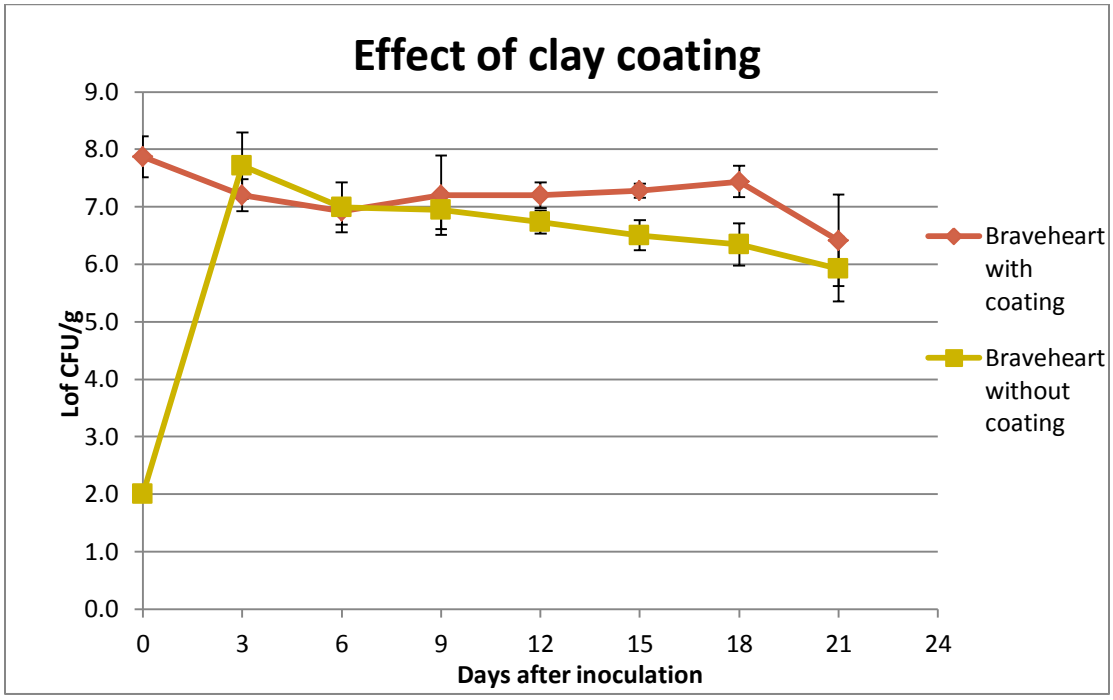

Figure 10: Effect of presence of seed clay coating on persistence of *L. monocytogenes* for a 21 day period (grown in sterile test tubes). Data points for each day reported are based on the average CFU/g values of 5 replicates. Error bars denote standard deviations.

All romaine seeds were provided by a commercial grower that uses seeds that have been coated in clay to allow for uniform sizing since planting is usually done via mechanized precision planters. Hence, it was important to test whether the presence of this clay coating was responsible for a greater retention of *L. monocytogenes* (Figure 10). Seeds of the same Braveheart cultivar that were

uncoated and in their original form were also obtained. As with other trials, Day 0 values were not taken into account due to the large variability across all trials. No statistically significant differences were found for any of the 21 days between the 2 growth curves (Fig. 10). Therefore, the presence of the clay coating does not affect the persistence of *L. monocytogenes* on romaine lettuce during a seed contamination event.

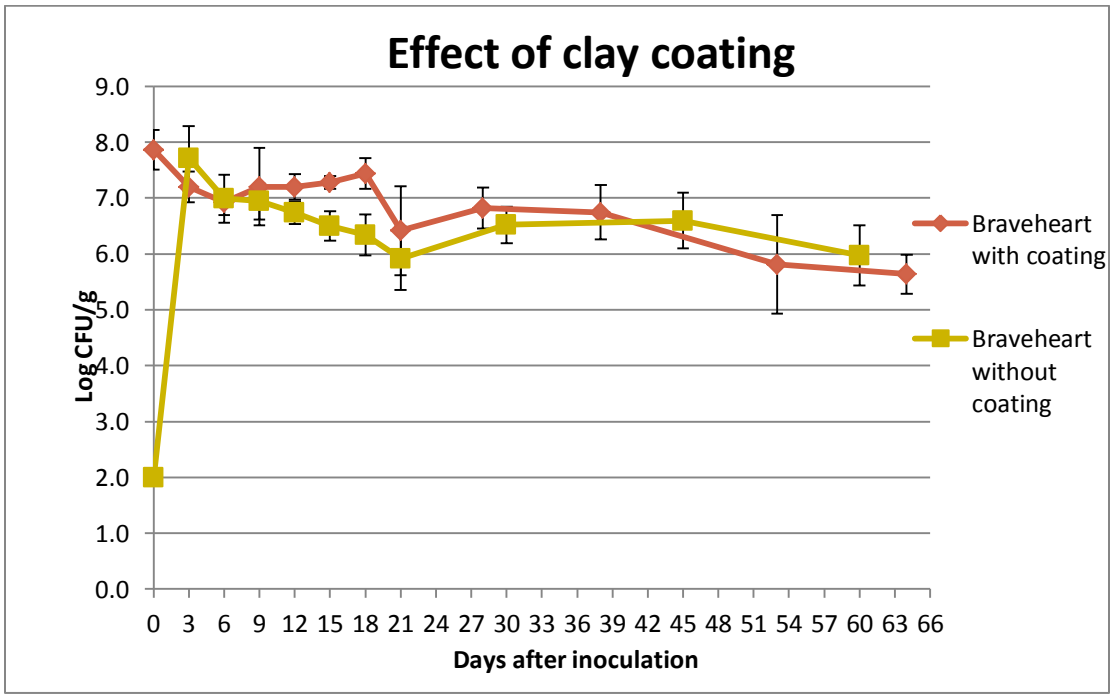

Figure 11: Effect of presence of seed clay coating on persistence of *L. monocytogenes* throughout the trial (grown in sterile test tubes). Data points for each day reported are based on the average CFU/g values of 5 replicates. Error bars denote standard deviations.

When sampling times were extended, both seemed to follow a similar trajectory with most *L. monocytogenes* 10403S enumeration values being within a close range (Figure 11). Final data points around the 60 day period were 5.64 log CFU/g for plants grown from clay coated seeds and 5.96 log CFU/g for plants grown from uncoated seed (Fig. 11). Hence, it is unlikely that the presence or absence of a coating contributes to any differences in *L. monocytogenes* persistence, even through to the harvest period.

2.4.4 Determining presence of *L. monocytogenes* in potting mix or soil used for non-test tube trials

Potting mix used in Trials 9 and 10 was plated without enrichment and had approximately 4.0×10^1 CFU/g of background microflora. This included a variety of different types of bacterial colonies and even some mold. When the potting mix sample was enriched with BLEB and LSES, and plated on MOX, there were fewer types of background microflora present due to the selective pressure during enrichment. A representative portion of the colonies present were streaked onto LMPM to assess actual presence of *L. monocytogenes*. However, none of the colonies streaked on LMPM showed the characteristic teal coloration that is seen with *L. monocytogenes* colonies that are able to hydrolyze the chromogenic media substrate due to presence of enzyme phospholipase C. Hence, no *L. monocytogenes* was detected from the enrichment.

The 75:25 mixture of Indiana top soil and potting mix was also assessed for *L. monocytogenes* growth. The non-enriched sample showed about 4.0 x 10^4 CFU/g of background growth with a variety of microbial colonies. This sample was enriched with BLEB, LSES and plated on MOX as well. As with the potting mix, none of the colonies that were restreaked on LMPM showed positive *L. monocytogenes.* Therefore, there was no *L. monocytogenes* detected in the 75% Indiana top soil mixture.

2.5 Discussion

In order to understand the ability of *L. monocytogenes* to persist on romaine lettuce, seeds from 3 cultivars were independently inoculated with 3 different strains of the pathogen and were grown in various types of growth media. This growth curve study found that under sterile conditions, romaine lettuce plants are able to support the growth of *L. monocytogenes* at relatively high levels for an extended period of time. In order to study the effect of strain differences on a single cultivar of romaine lettuce, *L. monocytogenes* strains FSL J1-208, FSL J1-194, and 10403S were used. Each of the strains is of a different serotype and lineage. FSL J1-208 is an isolate from an animal clinical case with serotype 4a and belongs to lineage IV. FSL J1-194 is an isolate from a sporadic human listeriosis case with serotype 1/2b and belongs to lineage I. *L. monocytogenes* 10403S is a serotype 1/2a strain isolated from a human skin lesion and is of lineage II (Broad Institute, 2010). The reason for

using these three strain was to test whether isolates typically found from human cases (lineage I), animal cases (lineage IV) or food (lineage II) had an advantage over the others in a plant environment. Based on the results obtained, there were no significant differences in the ability of the 3 strains to persist on romaine lettuce. This finding is in accordance with the results from other studies. Milillo et al. (2008) reported no differences in the abilities of 4 different strains of *L. monocytogenes*, each representing a different lineage, to grow on *Arabidopsis thaliana* leaves (Milillo et al., 2008). Additionally, while Gorski et al. (2004) found strain specific differences when testing the ability of *L. monocytogenes* to attach and grow on alfalfa sprouts, they did not find significant differences between lineages (Gorski et al., 2004).

Another parameter that was tested in this study was the influence specific cultivars have regarding the persistence of *L. monocytogenes* on romaine lettuce. Using strain 10403S, significant differences in cultivar persistence was only observed from Day 15 onwards out of the 21 day sampling period. While it is not possible to ascertain whether cultivar differences are significant past day 21, based on Fig. 3, it is quite evident that there is a large variation in persistence values between the Braveheart, Sunbelt and Sun Valley cultivars between days 21 to 60. Using strain FSL J1-194, there was no significant difference found between the cultivars during the first 21 day period. However, when extended to harvest period (Fig. 7) a more pronounced variation in persistence was again evident. Based on this data, it is entirely possible that cultivar differences are present but only manifest after an 18 to 21 day period in romaine lettuce. Another point to support this is that the Sunbelt

cultivar resulted in the highest persistence of the *L. monocytogenes* strain used, sometimes by even up to more than 2 log CFU/g higher than the Braveheart of Sun Valley cultivars (Fig. 3 and Fig. 7). The Sunbelt cultivar somehow provides a better microenvironment for the proliferation of *L. monocytogenes* perhaps via more protective sites or easier access to nutrients. Information such as this could be useful to the industry in order to be able to pick production cultivars that do not sustain pathogen growth as well. The influence of cultivar on the persistence of pathogens has been demonstrated before. Macarisin et al. (2013) showed that persistence of *E. coli* O157:H7 on spinach was significantly affected by cultivar characteristics (Macarisin et al., 2013). The four spinach cultivars the authors used had differences in leaf blade roughness and stoma density which they suggested contributed to the variation in persistence. It would be interesting to conduct a similar experiment as future work to determine the phenotypic differences between the 3 romaine lettuce cultivars used in this study.

Differences in the ability of *L. monocytogenes* to persist on or within the plants when grown in the various growth media were also evaluated (Fig. 8, 9). *L. monocytogenes* was found to persist the best in sterile soft-top agar while falling to below the limit of detection (2 log CFU/g) in all soil or potting mix (Sun Gro Sunshine Redi-Earth) trials conducted during a full harvest period. The soft-top agar trials provide a sterile environment in which the only bacteria present, other than the inoculated *L. monocytogenes*, is what was originally present on the seed when planted. There is no exchange of microflora with the environment as is present with

the romaine lettuce grown in the soil trials. The presence of soil microflora may therefore provide higher levels of competition to pathogens compared to the plant microflora alone. Supporting this idea is the fact that higher levels of soil microflora, and therefore competition, were found in the 75% Indiana top soil mixture than in the commercial potting mix and this could have explained why *L. monocytogenes* persistence levels dropped to undetectable levels by Day 18 in the top soil mixture versus Day 45 in the commercial potting mix. Bacterial competition could take the form of competition for nutrients, attachment sites or even the ability to change the microenvironment by altering pH or producing antimicrobials. The action of competitive soil microflora in reducing detectable pathogen numbers compared to other relatively less microbial-rich media has been noted in other studies as well (Cooley et al., 2003, Klerks et al., 2007, Warriner et al., 2003). However, following this logic, the results should have shown that *L. monocytogenes* persisted the longest in autoclaved potting mix versus in commercial potting mix or 75% Indiana top soil. The results showed otherwise with persistence on autoclaved potting mix being shorter than on unautoclaved potting mix. Commercial potting mixes usually contain some combination of chemical fertilizers or other additives such as wetting agents. Perhaps these compounds were inactivated when autoclaved and lost their functionality, resulting in the plants not being able to provide *L. monocytogenes* with as advantageous a colonization as the plant grown in unautoclaved potting mix did. There have been studies describing changes in chemical properties, such as pH, or even in physical properties, such as the development of water repellency in

intermediate moisture soils when autoclaved (Urbanek et al., 2010, Wolf et al., 1989). Perhaps a similar situation happened in this case but since these parameters were not tested, the true reason remains unknown.

In addition to differences in levels of competitive bacteria, the differences in relative humidity between sterile tube trials and soil trials should be noted. Relative humidity within the enclosed sterile test tubes is likely extremely high all the time while in the greenhouse it fluctuates at a lower level for the most part due to ventilation capabilities. Bacteria have been shown to proliferate better at a high relative humidity and this factor could also help explain, in part, why persistence of the pathogen in sterile tubes was much higher (Barak et al., 2011, Leben, 1988). The romaine lettuce plants grown in sterile tubes, however, did not grow as well as the plants from seeds that were planted in soil or potting mix. It was determined that the space provided by the 25x200mm test tubes was not as issue since seeds that were planted in much larger magenta boxes (77 mm × 77 mm × 97 mm) as a test showed similar growth (data not shown). The lack of nutrients in the soft-top agar combined with the high initial pathogen load could have contributed to this.

The Day 0 *L. monocytogenes* concentrations on the various cultivars of romaine lettuce were not included in the statistical analysis since some trials resulted in counts that were lower than the limit of detection. The lack of recovery of the pathogen in some of the trials could possibly be explained due to *L. monocytogenes* cells being subjected to stress or shock via the washing, centrifuging, rotating and drying on seed steps before being sampled. Additionally, the trials that

resulted in concentrations below the limit of detection were found to belong to either the Sunbelt cultivar (regardless of whether it was inoculated with *L. monocytogenes* 10403S or FSL J1-194) or the seeds that were specifically ordered without the typical clay coating. This is significant because during seed contamination with the Sunbelt seeds, the coatings washed off and the pathogen would have had to attach directly to the seed, just as with the seeds ordered without a coating. Also, the differences in concentrations of *L. monocytogenes* on the other trials that were measurable on Day 0 could have been attributed to differences in moisture content of the clay coatings or even the seed itself. For instance, if seeds were drier to begin with, then they would imbibe more of the initial concentration and potentially have higher concentrations of the pathogen versus seeds that already have higher moisture content and imbibe less water. Regardless of Day 0 counts, however, all trials within their comparison groups (Fig. 2 to Fig. 11) recovered to similar log CFU/g values by Day 3. Hence, the remainder of the period of persistence studies for all the trials should have been representative of any associations found.

There have been other studies that have used similar methods of seed inoculation to test pathogen persistence. While it is possible that seeds could be contaminated from soil or water sources in a field, it is also possible that they could become contaminated in a different manner prior to being planted in an otherwise pathogen free area. Van der Linden et al. (2013) have shown that butterhead lettuce seeds inoculated with *E. coli* O157:H7 and *S. enterica* still tested positive for the

respective pathogens when sampled two years after storage. The two year old contaminated seeds were then germinated and pathogen populations reached 5.92 log CFU/seedling for *Salmonella* serovars and 4.41 log CFU/seedling for *E. coli* respectively (Van der Linden et al., 2013). This shows that seeds do not necessarily have to be contaminated at the point right before or during germination. Pathogen inoculation on seeds from a variety of produce types such as carrot, spinach, radish, tomato, cress and lettuce was studied. The pathogens used included *E. coli* O157:H7, *S. enterica* and *L. monocytogenes*. Interestingly, *L. monocytogenes* numbers on germinating seeds were much higher than the other two pathogens even though all were inoculated at a concentration of 2 log CFU/ml. After 49 days of growth, *L. monocytogenes* was found to persist on cress at 5.89 log CFU/g, on radish at 3.33 log CFU/g, on spinach at 5.33 log CFU/g and on lettuce at 5.91 log CFU/g (Jablasone et al., 2005). These studies, in concordance with this study, highlights that contaminated plants can arise from contaminated lettuce seeds and that bacterial pathogens are able to persist on them.

Overall, this study has shown that even a brief contamination period of 30 minutes can result in *L. monocytogenes* attachment to seeds and its growth and persistence up to the harvest period of the mature plant under ideal conditions. Based on the results of this study, *L. monocytogenes* is unlikely to be able to persist until the harvest period of romaine lettuce plants grown from contaminated seed in a soil or potting mix environment. However, with so many factors such as soil type, climate and diversity of competitive microflora all playing an essential role in

pathogen persistence, it is difficult to draw generalized conclusions based on the relatively small subset of factors tested. It is possible that a combination of ideal factors may allow persistence until harvest under field conditions. Additionally, germination of a contaminated seed allows for conditions that may prove to be suitable for pathogen proliferation since germination requires a moist and nutrient rich environment (Erickson et al., 2014). Hence, reducing the exposure of seeds to conditions under which they could become contaminated, regardless of whether its use in planting is imminent of not, is important. It is also crucial that the industry continues to take adequate measures in the prevention of contamination such as those outlined by GAPs. Care should be taken in ensuring that irrigation water are of appropriate microbial quality is used, that any soil amendments used have been treated or properly composted, and that seed storage prior to use has been under appropriate conditions. Regardless, the understanding of *L. monocytogenes'* ability to persist on romaine lettuce may aid in further understanding in determining the level of sanitizing efficacy required during post-harvest processing, or even in cross contamination risk assessment models based on pathogen load.

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CHAPTER 3. INTERNALIZATION OF *LISTERIA MONOCYTOGENES* IN ROMAINE LETTUCE

3.1 Abstract

Internalization of pathogens in a variety of produce types has been demonstrated, but few focus on *Listeria monocytogenes* in pre-harvest conditions. In this study, the internalization potential of *L. monocytogenes* in 20 day old romaine lettuce plants was assessed. Seeds were contaminated with *L. monocytogenes* that constitutively expressed green fluorescent protein (GFP) and were grown in a greenhouse setting. Three plant replicates were fixed, paraffin embedded, and sectioned into 10 µm longitudinal sections. All sections were visualized using fluorescence microscopy following detection of each bacterium present in the tissue using immunohistochemistry techniques with GFP specific antibodies. Results showed that a total of 539 *L. monocytogenes* cells were observed within the hypocotyl sections and were found to be localized in every major tissue type. The highest incidence of *L. monocytogenes* (34.1%) was found in the pith tissue which is the innermost tissue of the stem. The pathogen was also present in the plant vasculature which indicates its potential to be transported throughout the plant. In addition, these data indicate that *L. monocytogenes* does not seem to internalize

according to known mechanisms due to majority of the bacteria being in the apoplast. Overall, these results show that *L. monocytogenes* can internalize in romaine lettuce and that it can potentially serve as a vehicle of transmission to consumers. Confirmation of internalization also indicates that current surface sanitization treatments are most likely inadequate as a food safety strategy.

3.2 Introduction

There have been numerous studies within the past 10 years that have conducted pathogen internalization experiments. Overall, the studies have shown a wide range of results from successful internalization (Deering et al., 2011, Deering et al., 2012, Solomon and Matthews, 2005) to occasional cases of internalization (Bernstein et al., 2007, Jablasone et al., 2005), to failure of internalization altogether (Mitra et al., 2009, , Zhang et al., 2009). The wide range of results is potentially due not only to the different inoculation and detection methods used, but also differences among strains, produce type and interactions between them (Olaimat and Holley, 2012). There are several ways in which pathogens can infiltrate and internalize in plant tissue. Damaged tissue can be a site of entry which was shown when leaf tip burn lesions, a lettuce disorder generally caused by inadequate calcium uptake, were shown to harbor high populations of *E. coli* O157:H7 both externally and internally (Brandl, 2008). Tissue damage can also take the form of a tear in the plant cuticle which is the waxy, protective layer found on the epidermis.

Healthy tissue can also act as a potential internalization site especially with regards to stomata and trichomes. Stomata are small pores on the surface of leaves and stems whose opening and closing are regulated by guard cells (shown in Figure 12). Stomata are involved in transpiration of water and gas exchange in plants (Erickson, 2012). While invasion of the stomata and sub-stomal cavity itself may not be considered true internalization per se, Kroupitski et al. showed that when incubated in light conditions, leaf inoculated *Salmonella enterica* not only occupied the stomatal cavity, but also internalized into the inner leaf tissue, specifically the apoplast of the spongy parenchyma (Kroupitski et al., 2009). Yet another path for internalization of pathogens is via germinating seeds. Pathogens that are present in the area of germinating roots and radicals are exposed to nutritional exudates from the growing plant which could cause the pathogens to proliferate in its immediate vicinity and gain access to the plant. This will be discussed further in later sections. *S. enterica* serovar Montevideo and serovar Michigan were also found internalized in the seedlings of tomato plants that were hydroponically grown in contaminated nutrient solution (Guo et al., 2002). The fact that the pathogen was found in the hypocotyl, cotyledons, stems and leaves of the seedlings demonstrates that pathogens can internalize in a plant via uptake of water and subsequent systemic transport of the pathogen through the plant is possible.

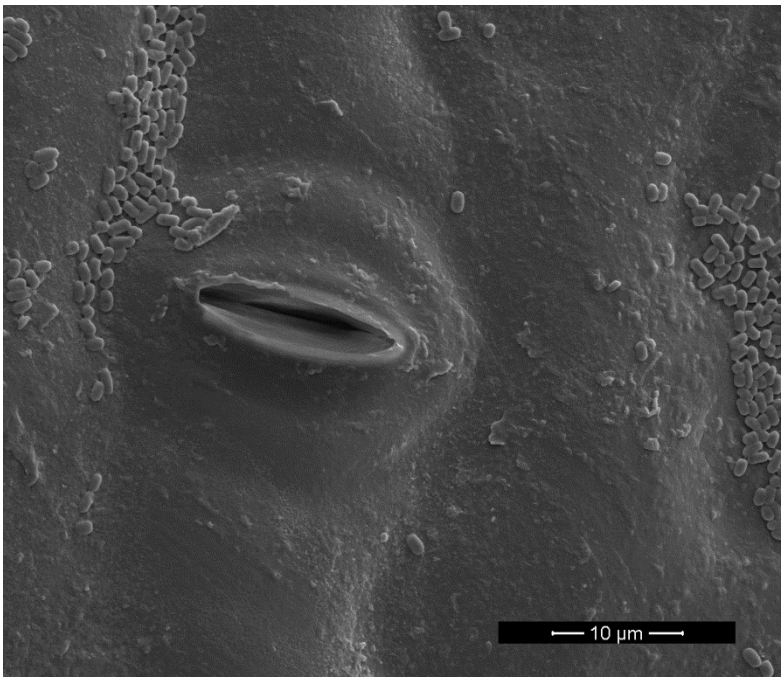

Figure 12: Cryo-SEM image of leaf stomata surrounded by bacteria on romaine lettuce (provided by A. Deering).

Internalization of pathogens in a variety of produce types has been demonstrated. Goldberg et al. (2011) showed leaf internalization of *S.* Typhimurium varied in different types of produce based on the percentage of 30 microscopic fields examined. Internalization was found to be highest in arugula (88%) followed by iceberg lettuce (81%), basil (46%), red lettuce (20%), romaine lettuce (16%), parsley (2%) and tomato (0.6%) (Golberg et al., 2011). With regards to lettuce alone, internalization potential of *E. coli* O157:H7 (Franz et al., 2007, Mootian et al., 2009, Nthenge et al., 2007, Solomon et al., 2002a), murine norovirus (Wei et al., 2010), canine calcivirus (Urbanucci et al., 2009) and various serovars of *S. enterica (Bernstein et al., 2007, Franz et al., 2007, Klerks et al., 2007)* have been demonstrated. It is clear that not only are there a variety of routes of entry for

pathogens into plants, but also that efficacy of internalization is in part determined by produce type and even pathogen species.

Internalization of pathogens in produce is definitely a large concern for the food industry. Current sanitization practices are largely focused on reducing surface bacteria. New sanitization practices or technologies need to be investigated in order to address the possibility that produce could be carrying internalized bacteria. Perhaps, if prevention is truly better than cure, more attention needs to be paid to preventing pre-harvest contamination rather than trying to find ways to sanitize internalized bacteria since such methods are more than likely to affect the quality of produce. Pathogen internalization is problematic especially since consumption of certain produce items, such as lettuce, is often done without a “kill step” or a thermal process to inactivate the pathogen.

There are various methods that have been used to study the internalization of pathogens. One of the more simple approaches is to contaminate a specific part of the plant and after a period of time, to test for the pathogen at a different part of the plant. The idea being that any positive results from the pathogen at the second site would be via transfer through the plant system. While there are quite a few studies that have used this method (Guo et al., 2002, Habteselassie et al., 2010, Solomon and Matthews, 2005), care has to be taken in these experiments so as to not inadvertently transfer the pathogen by direct contact or splashing when watering or even through possible aerosolization of contaminated soil particles. Additionally, it is possible that the target pathogens were able to move from one

portion of the plant to another via capillary action of water on the outer surfaces of the plant without having to be internalization and move through the plant system (Erickson, 2012). Another method, and one of the most common, is that of surface sterilization. In this method, a disinfectant or sanitizer is used to sterilize the outer surface of the plant following contamination and the resulting surface-sterilized plant is enumerated with the idea that any bacteria obtained would have been internalized. Enumeration is conducted by grinding and plating the surface sterilized plant. While it makes sense theoretically, there are a number of issues with such a method. First, sanitizers may not be 100% efficient due to the fact that target pathogens could be incorporated into biofilms that may aid in resistance to the sanitizer. This could lead to false positives. On the contrary, if the sanitizer was somehow pulled into the inner tissue of the plant, enumeration could result in an underestimation of the internalized pathogens should they really be there (Erickson, 2012). Yet another issue with this method is that since bacteria have been shown to enter stomatal openings on the leaf (Berger et al., 2010), any bacteria in the sub-stomatal cavity when the guard cells close will evade any sort of sanitizing treatment (Seo and Frank, 1999). Lastly, it is not possible to tell the specific location of internalization with this method.

Another method to study internalization is via the use of cell labelling techniques which are then usually performed in combination with microscopy. One of these cell labelling techniques involves visualizing the β-glucuronidase (GUS) enzyme activity. The *gus* gene, originally isolated from *E. coli*, is not typically found

in plants or in a wide range of bacteria which makes it suitable for use in plant studies if inserted into target bacterium (Wilson et al., 1995). When incubated with its chromogenic substrate, presence of GUS activity causes the cleavage of said substrate and results in an accumulation of a blue precipitate which can be visualized (Jongen, 2005). GUS assays allow for both quantitative analysis as well as localization analysis for the target bacterium (Wilson et al., 1995). The obvious downside to this method is that it cannot be used if the target bacterium, especially if studying *E. coli*, or any other bacteria that may be present and have the *gus* gene in its genome. The other reporter gene that is commonly used is *gfp* which produces the green fluorescent protein (GFP). GFP fluoresces under ultraviolet light without needing any additional cellular cofactors or energy. The *gfp* gene is often inserted into a plasmid that is contained within the target host bacterium. The disadvantage to using plasmids, however, is that they can often be lost if there is no selective pressure used to maintain them (Warriner et al., 2003). For this reason, any experiments involving studying plant and pathogen interaction over extended periods of time cannot use plasmid vectors. For example, Jablasone et al. (2005) attempted to use pathogens containing GFP labeled plasmids to track their interactions with growing plants. While they managed to achieve plasmid stability with *Salmonella* and *E. coli* O157:H7, the plasmid label inserted into *L. monocytogenes* was lost after only one subculture and the authors were unable to track that interaction (Jablasone et al., 2005). Additionally, selective agents cannot be used reliably or efficiently in plant pathogen experiments.

Reporter gene products sometimes require analysis via microscopy to be visualized. This allows for a far more detailed analysis of localization and quantification by tissue type. The most common type of microscopy used is confocal microscopy. Laser scanning confocal microscopy (LSCM) uses optical sectioning to render a three dimensional image of the object being studied (Erickson, 2012). While this allows increased optical resolution and contrast, the disadvantage to using this method of microscopy is that fluorescent signals have the potential to bleed from one section to another. This may result in a positive signal being registered in multiple locations even though it is really only present in one optical section (Deering et al., 2012). Additionally, the low sensitivity of LSCM in the detection of GFP-labelled pathogens requires that high numbers of the target pathogen be present in order to be visualized (Warriner et al., 2003). The obvious limitation of this method is that should internalized labelled pathogen numbers drop to low levels, the pathogen has the potential to go undetected and a lack of internalization could be recorded. Epifluorescence microscopy that is used for direct visualization of GFP can also be problematic. This is because plant tissue cannot be treated for preservation or fixed since such a treatment would result in loss of fluorescence of GFP. Hence, fresh tissue needs to be used. Unlike LSCM, epifluorescence microscopy necessitates sectioning of the plant tissue. When fresh tissue is used, slicing sections can cause bacteria to be dislodged from its original position and move to a different position in the tissue (Deering et al., 2012). For example, target bacterium on the plant surface could be pulled into the inner tissue by the blade. This confounds the

reliability of any internalization results obtained in such a manner. While not as common, both scanning electron microscopy (SEM) and transmission electron microscopy (TEM) have been used to study internalization of pathogens (Itoh et al., 1998, Jablasone et al., 2005, Janes et al., 2005, Standing et al., 2013). While providing high level resolution, TEM would be an arduous task for experiments designed to study multiple replicates of plant tissue or large areas of tissue since it requires ultrathin sections of the tissue to be prepared. SEM, on the other hand, does not require true sections of plant tissue but requires the sample to be coated in conductive material and depending on the material's thickness, can interfere with the observations of cellular details (Wilson and Bacic, 2012). Additionally, since SEM looks at surface topography, one would not be able to conduct internalization studies unless the internal portions of the plant are somehow exposed. This typically involves freeze fracturing the tissue sample, but with no way of really controlling the direction of the fracture plane, may hinder proper analysis (Walther and Müller, 1999). In addition, this would only enable determination of presence or absence of bacteria in the area exposed via freeze fracturing and not that of the whole plant.

The objective of this study was to determine the potential for *L. monocytogenes* to internalize in romaine lettuce. This was tested by inoculating romaine lettuce seeds with *L. monocytogenes* constitutively expressing GFP and growing them in a greenhouse setting. Harvested 20 day old plant stems were fixed, sectioned and subjected to standard immunohistochemistry techniques. Slides of sectioned romaine hypocotyl tissue were analyzed via fluorescence microscopy in

order to visualize any internalized *L. monocytogenes* bacteria as well as to assess localization patterns if present.

3.3 Materials and Methods

3.3.1 Construction of *Listeria monocytogenes*-GFP isolates

Listeria monocytogenes strain FSL B2-107 (10403S background with *gfp* gene chromosomally inserted into the $tRNA^{Arg}$ locus via pH-*hly gfp*-PL3) was kindly donated by the Food Safety Laboratory at Cornell. Only the other two isolates used, FSL J1-194 and FSL J1-208, required GFP constructs inserted into them.

The plasmid, pH-*hly gfp*-PL3, hosted in an XL1-Blue strain of *Escherichia coli* was generously donated by Dr. Higgins, Harvard University. An overnight culture of *E. coli* hosting the plasmid was grown in Luria-Bertani (LB; 0.01g/ml tryptone, 0.005g/ml yeast extract, 0.01g/ml sodium chloride) broth with 20 µg/ml chloramphenicol and shaking at 37°C, 200 rpm. Plasmid extraction was accomplished using Qiagen® plasmid Mini kit (QIAGEN, Inc., Valencia, CA). Final eluted plasmid was suspended in 20 µl of TE buffer, pH 8.0.

To make competent *Listeria monocytogenes* cells, an overnight culture of each of the other two isolates, FSL J1-194 and FSL J1-208, were grown in 5ml of BHI broth with shaking at 200 rpm at 37°C. From this overnight culture, 0.5 ml was used to inoculate 50 ml BHI with 0.5M sucrose which was shaken at 37°C until OD_{600} = 0.2. Penicillin G was added to the culture to give a final concentration of 10 µg/ml,

followed by shaking at 37°C for 2 hours. The culture was centrifuged at 4°C at 7000 rpm for 10 min. The pellet was resuspended in a 45ml HEPES solution (1mM HEPES (pH 7), 0.5M sucrose, 10% glycerol). Centrifuging at the same 4°C parameters was repeated two more times but with 22.5ml and 0.4ml of the HEPES solution respectively. Competent cells were promptly used in the following electroporation steps.

In a 0.1cm cuvette, 910 ng (in a volume of 10 µl) of plasmid was used for 100 µl of *Listeria monocytogenes.* Electroporation was carried out in a Gene Pulser® (Bio-Rad Laboratories, Hercules, CA) at a voltage 1400V, resistance of 100Ω and capacitance of 25F. Immediately following this, 1 ml of a BHI with 0.5 M sucrose solution was added, placed on ice for 30 seconds and further incubated with shaking at 30° C for 1.5 hours. Incubated culture of electroporated cells was then spread plated in 100µl volumes onto BHI with 7.5 µg/ml chloramphenicol selection plates. Plates were incubated for 3 days at 30° C. Primers NC16 (5'-TCAAAACATACGCTCTTATC) and PL95 (5'-ACATAATCAGTCCAAAGTAGATGC; Intergrated DNA Technologies, Coralville, Iowa) were used to check integration by amplifying across the $tRNA^{Arg}$-*attBP'* hybrid attachment site.

Freezer stocks of these two new integrated GFP *L. monocytogenes* strains were stored at -80°C in Brain-Heart Infusion (BHI) (Bacto™, Sparks, MD) broth supplemented with 25% glycerol. These isolates were stored for future use while the original FSL B2-107 (10403S background strain with GFP) was used for further immunohistochemistry experiments.

3.3.2 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Western Blot specificity and optimization for GFP

Whole cell protein extraction was done from an overnight culture of FSL B2-107 (10403S background with GFP). A 6 ml culture volume was spun down at 6000 rpm for 6 minutes. Pellets were washed twice with phosphate buffer saline solution (9mg/ml sodium chloride in 100 ml 0.1 M phosphate buffer) and resuspended in 200µl sample solvent (46mg/ml sodium dodecyl sulfate (SDS); 20% glycerol; 15mg/ml tris base; 10% beta-mercaptoethanol; sterile water to 20 ml). The sample was boiled for 10 minutes followed by an additional 200 µl sample solvent added to the sample and continuous sonication 4 times for 30 seconds each. Next, the samples centrifuged for 10 minutes at 12000 rpm and the supernatant containing whole cells proteins was collected. Prior to running the SDS gel, 5µl of 10% bromophenol blue was added to the samples and boiled for 10 minutes.

For separation of proteins by SDS-PAGE, 7 different samples of whole cell protein extract from FSL B2-107 were loaded into 4% (stacking gel) and 12% (separating gel) Bis-tris gels. A volume of 12µl was loaded for each sample and 10µl of pre-stained SDS-PAGE broad range standard (Bio-Rad Laboratories, Hercules, CA) was used as a ladder. Gels were run in 1x running buffer (25mM tris base; 250mM glycine; 0.1% SDS) at 120V at room temperature. Gels were removed, and protein was transferred overnight in a mini trans-blot cell (Bio-Rad Laboratories, Hercules, CA) to a polyvinylidene-difluoride (PVDF) membrane at 55V, 4°C in 1x transfer buffer (47mM tris base; 38mM glycine; 0.04% SDS; 20% methanol).

A blocking agent of 4% non-fat dry milk in Tris buffer saline solution with Tween 20 (TBST; 150mM sodium chloride, 20mM tris base, 0.05% Tween 20, pH 7.5) was added to the blot at room temperature for 2 hours with rotation. This step was followed by incubation with a 1:1000 dilution of the primary anti-GFP antibody (Living Colors® A.v. peptide antibody, affinity purified rabbit immunoglobulin G, Clontech laboratories, Inc. Mountain View, CA) in 0.5x blocking buffer for 2 hours at room temperature with rotation. The blot was washed 3 times for 10 minutes each with TBST pH 7.5 before being incubated with a 1:2500 dilution of the secondary alkaline phosphatase conjugated antibody (anti-rabbit immunoglobin G, Sigma, St.Louis, MO), also in 0.5x blocking buffer for 2 hours at room temperature with rotation. The blot was once again removed and washed 3 times for 10 minutes each with TBST pH 7.5. Approximately 10ml of substrate for alkaline phosphatase (Western Blue®, Promega, Madison, WI) was added to the blot and allowed to develop at room temperature until bands were visualized. The reaction was stopped after a period of 5 minutes by rinsing the blot with distilled water.

3.3.3 SDS-PAGE, Western blot check for cross-reaction with plant tissue

Total plant protein was extracted from non-inoculated plants grown for 30 days in soil in the greenhouse. Two separate plants were used and sections of root, stem and leaf were removed from each. Sections were ground in an Eppendorf tube with 250µl of 1x sample buffer with DL-dithiothreitol (DTT) (Sample buffer; 50mM tris-HCl pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol, 100mM

dithiothreitol). Samples were boiled for 12 minutes and then centrifuged for 3 minutes at 11000 rpm.

For separation of proteins by SDS-PAGE, 6 different samples of total protein extract from the duplicate stem, root, leaf sections were loaded into 4% (stacking gel) and 12% (separating gel) Bis-tris gels. A volume of 12µl was loaded for each sample and 10µl of Precision Plus Protein™ All Blue standard (Bio-Rad Laboratories, Hercules, CA) was used as a ladder. A positive control of whole cell protein extract from FSL B2-107-GFP was also loaded into the gel. Gels were run in 1x running buffer (25mM tris base; 250mM glycine; 0.1% SDS) at 120V at room temperature. Gels were removed, and protein was transferred overnight in a mini trans-blot cell to a polyvinylidene difluoride (PVDF) membrane at 55V, 4°C in 1x transfer buffer (47mM tris base; 38mM glycine; 0.04% SDS; 20% methanol).

A blocking agent of 4% non-fat dairy milk in tris buffer saline solution with TBST (TBST; 150mM sodium chloride, 20mM tris base, 0.05% Tween 20, pH 7.5) was added to the blot at room temperature for 2 hours with rotation. This step was followed by incubation with a 1:1000 dilution of the primary anti-GFP antibody (Living Colors® A.v. peptide antibody, affinity purified rabbit immunoglobulin G, Clontech laboratories, Inc. Mountain View, CA) in 0.5x blocking buffer for 2 hours at room temperature with rotation. The blot was washed 3 times for 10 minutes each with TBST pH7.5 before being incubated with a 1:2500 dilution of the secondary alkaline phosphatase conjugated antibody (anti-rabbit immunoglobin G, Sigma, St.Louis, MO), also in 0.5x blocking buffer for 2 hours at room temperature with

rotation. The blot was once again removed and washed 3 times for 10 minutes each with TBST pH7.5. About 10ml of substrate for alkaline phosphatase (Western Blue®, Promega, Madison, WI) was added to the blot and left to develop at room temperature until bands were visualized. The reaction was stopped after a period of 5 minutes by rinsing the blot with distilled water.

3.3.4 Plant tissue fixation and paraffin wax embedding

Braveheart cultivar seeds were inoculated with *L. monocytogenes* (FSL B2-107), planted in commercial potting mix and placed in the greenhouse as describe above. Plants were harvested after a period of 30 days and hypocotyl sections were cut from each of 25 plants. The stem sections were placed in a fixative solution (4% formaldehyde, 0.5% gluteraldehyde in 0.1M phosphate buffer pH 7.0) overnight at 4°C with rotation. The following day, sections were placed under a vacuum intermittently for 2 hours to remove any remaining air pockets and to ensure the fixative was evenly distributed throughout the tissue. Sections were then washed twice in 0.1M phosphate buffer pH 7.0 for one hour each before being dehydrated through a graded ethanol series (25%, 30%, 45%, 50% ethanol). Tert-butyl alcohol was used as the intermediate solvent between the ethanol series and a final infiltration of sections with liquid paraffin held at 58°C. The hypocotyl sections were then embedded in paraffin blocks and these were longitudinally sectioned into 10µm sections using a rotary microtome (Microm HM-330, Walldorf, Germany). The

sections were floated using water on microscope slides coated with 0.01% poly-L-lysine and dried overnight at 38°C.

3.3.5 Immunohistochemistry for *Listeria monocytogenes*-GFP detection

A total of approximately 910 paraffin sections placed on slides in sets of about 10 were obtained from 3 different plant stems that were sampled. Slides with paraffin sections were dewaxed in xylene twice for 10 minutes each. They were subsequently rehydrated through a graded ethanol series (100%, 95%, 75%, 50%, 30%) at 5 minute intervals. A hydrophobic well was placed around the sample using an ImmEdge™ pen (Vector Laboratories, Inc., Burlingame, CA). The slides were then washed with TBST pH 7.5 before being incubated in blocking buffer (4% bovine serum albumin, 0.1% triton-x in TBST) for 1 hour at room temperature in a humidity chamber. Once completed, the blocking buffer was removed and sections were briefly washed with TBST. A 1:100 dilution of the primary anti-GFP antibody (described above) in 0.5x blocking buffer was used to incubate sections for 1 hour at room temperature in a humidity chamber. Slides were washed 3 times for 10 minutes each with TBST and subsequently incubated in 1.25µl/ml goat anti-rabbit Alexa Fluor®568 secondary antibody (Invitrogen, Carlsbad, CA) in 0.5x blocking buffer. Incubation was done in the dark with the secondary antibody for 1 hour at room temperature in a humidity chamber. The slides were then washed 3 times for 10 minutes each with TBST and 3 drops of Fluoromount-G® mounting medium (Electron Microscopy Sciences, Hatfield, PA) were placed on each slide before a

coverslip was added. Slides were stored at 4°C in the dark until ready to be examined.

3.3.6 Microscopy

The hypocotyl sections from 3 different plants that were previously fixed in paraffin were examined using a Nikon® Microphot-FXA fluorescence microscope (Nikon, Melville, NY). Both fluorescence as well as differential interface contrast (DIC) images were used to examine the slides. Pictures were taken using a MicroFire® digital camera (Optronics, Goleta, CA). For each positively identified bacterium, the following details were recorded: slide number, section number, general tissue location, and apoplastic or symplastic localization. In order to appropriately determine a positive signal, strict criteria was used. This included only counting fluorescence signals observed under the correct filter set (i.e. observing fluorescence only at the appropriate excitation and emission wavelengths), restricting the size of the signal to between approximately 1-1.5 µm in length (typical length of *L. monocytogenes*), and ensuring the shape of the signal was either strictly a rod or circular (cross-section of *L. monocytogenes*). Controls were used to ensure that signals were not present otherwise and this involved treating slides with blocking buffer only, primary antibody only, and secondary antibody only. Additional controls were done with plants that were treated exactly as described above except they were non-inoculated with the bacterium. These tissues were prepared for examination as described for the treatment tissues. Images were cropped using

Adobe Photoshop CC (Adobe Systems, San Jose, CA) and scale bars were added using ImageJ (NIH, Bethesda, MD). *L. monocytogenes* concentration in plant tissue (bacteria/mm^3) was assessed by treating each hypocotyl as a cylinder and calculating the volume from the measured values of height and width. Localization patterns were analyzed using SAS 9.1 statistical software (SAS Institute Inc., Cary, NC). The Glimmix procedure (mixed model in which the Poisson distribution was used while taking into account plant random effects) was used for analysis. Tissue type and distance from the center of the hypocotyl were modeled as fixed effects. Differences in least square means that had been Tukey-Kramer adjusted for multiple comparisons were used to assess significant pair-wise comparisons of tissue type ($p<0.05$).

A single hypocotyl that had previously been fixed and embedded (as mentioned above) was used to identify the number of vascular bundles present in the romaine lettuce stem. Cross sections instead of longitudinal sections were obtained. After dewaxing and rehydrating steps were completed, 0.05% Toluidine blue was used to stain the slides for 10 minutes. Slides were then rinsed 3 times in 25% ethanol. Three drops of Permount™ mounting medium (Electron Microscopy Sciences, Hatfield, PA) were placed on each slide before a coverslip was added. Brightfield images were taken using the same MicroFire® digital camera.

3.4 Results

3.4.1 Construction of *Listeria monocytogenes*-GFP isolates

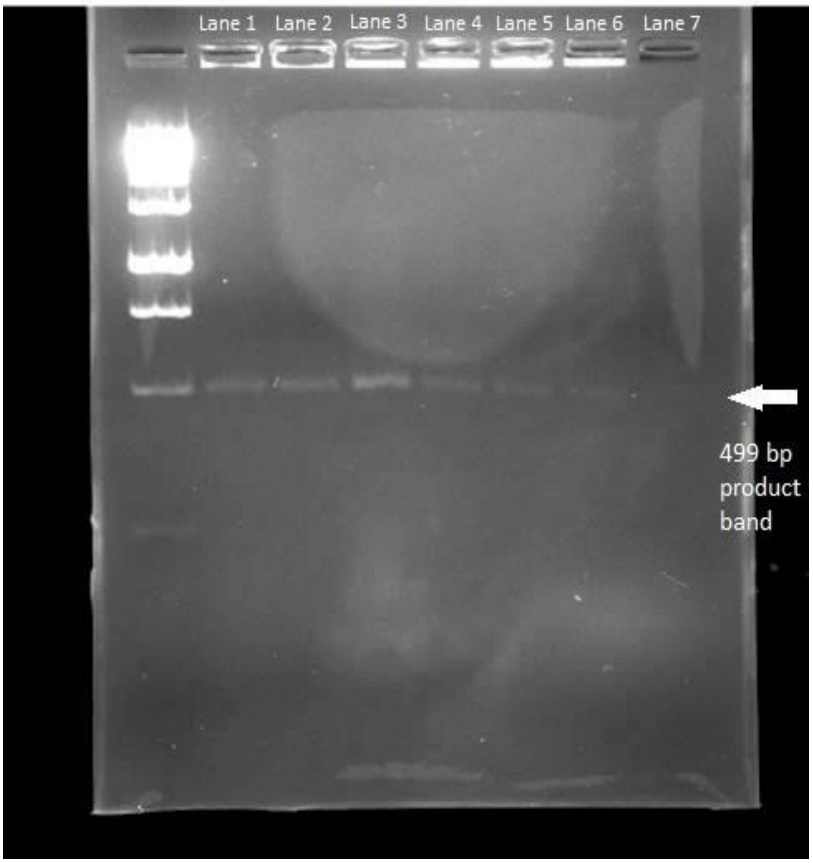

Figure 13: Gel electrophoresis image of pH-*hly gfp*-PL3 integrants. Lane 1: positive control FSL B2-107 (LM 10403S + GFP), Lane 2 to 5: different colonies of FSL J1-194 integrants, Lane 6: FSL J1-208 integrant, Lane 7: negative control.

Once electroporated and plated on antibiotic selection plates, any respective colonies of FSL J1-208 and FSL J1-194 that grew were analyzed via PCR to check for proper pH-*hly gfp*-PL3 integration (Fig. 13). A 499 bp product was expected and present across all lanes except the negative control lane. This indicates that pH-*hly*

gfp-PL3 successfully integrated into the $tRNA^{Arg}$ chromosomal region of the required strains.

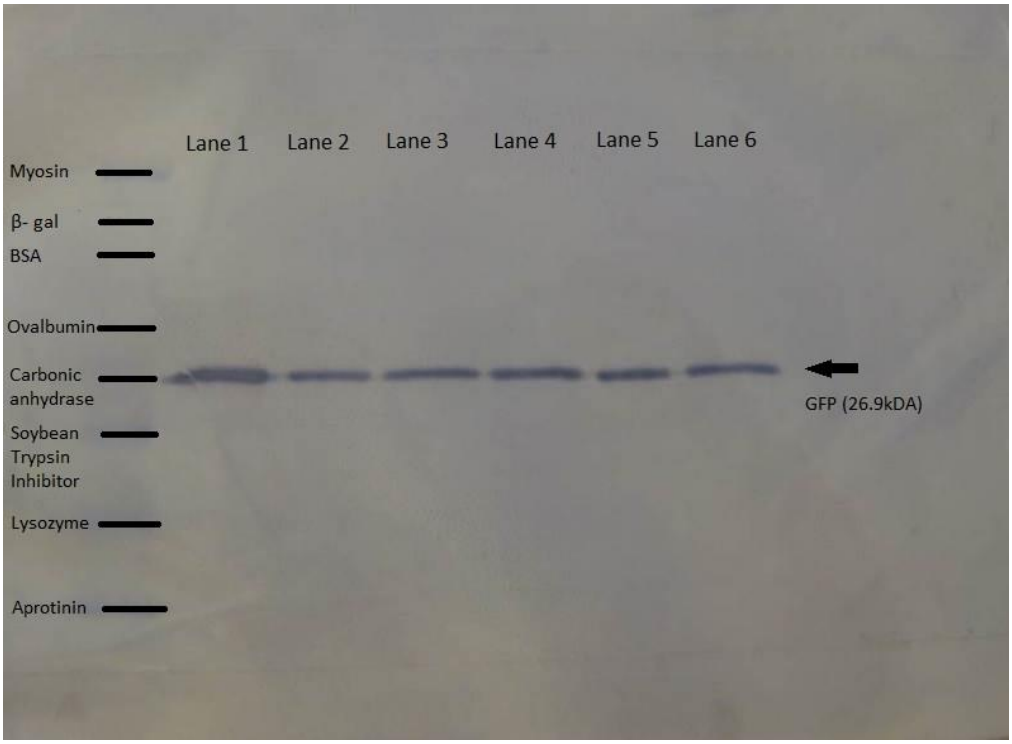

Figure 14: Western blot of pH-*hly gfp*-PL3 integrants. Lane 1: positive control FSL B2-107 (LM 10403S + GFP), Lane 2: FSL J1-208, Lane 3: FSL J1-208, Lane 4: FSL J1-194, Lane 5: FSL J1-194, Lane 6: FSL J1-194.

Total protein extracts from successful pH-*hly gfp*-PL3 integrants of FSL J1-208 and FSL J1-194 were analyzed to determine whether GFP was being expressed and if the level was comparable to that of the known GFP strain, FSL B2-107 (Fig. 14). SDS-PAGE was conducted with a 1:1000 1° antibody concentration and a 1:2500 2° antibody concentration as used in antibody optimization experiments. All samples

showed had GFP present and pH-*hly gfp*-PL3 integration was shown to be functional in the strains used.

3.4.2 SDS-PAGE, Western blot specificity and optimization for GFP

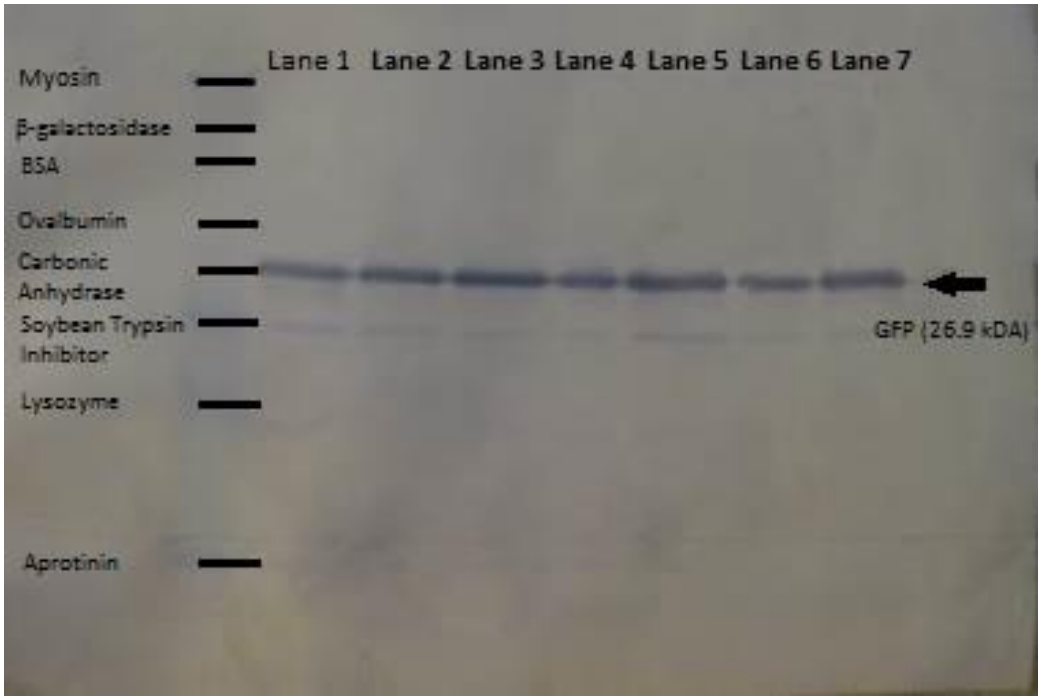

Figure 15: Western blot for optimization of 1° antibody concentration. Lane 1 to 7: All lanes are of undiluted protein extracts from separate cultures of the same strain FSL B2-107 (LM 10403S + GFP).

As shown in Figure 15, a 1:1000 1° antibody concentration was tested on protein extracts of FSL B2-107. All lanes show a band that is of comparable size to the carbonic anhydrase band in the marker. Carbonic anhydrase is 27.6 kDA in size while GFP is 26.9 kDA. The smaller faint bands under the main GFP band in each lane are likely that of GFP breakdown products. Different 1° antibody concentrations of

1:50, 1:100, 1:750 were tested prior but a concentration of 1: 1000 was found to give minimal to no background and was used for further experiments.

3.4.3 SDS-PAGE, Western blot check for cross-reaction with plant tissue

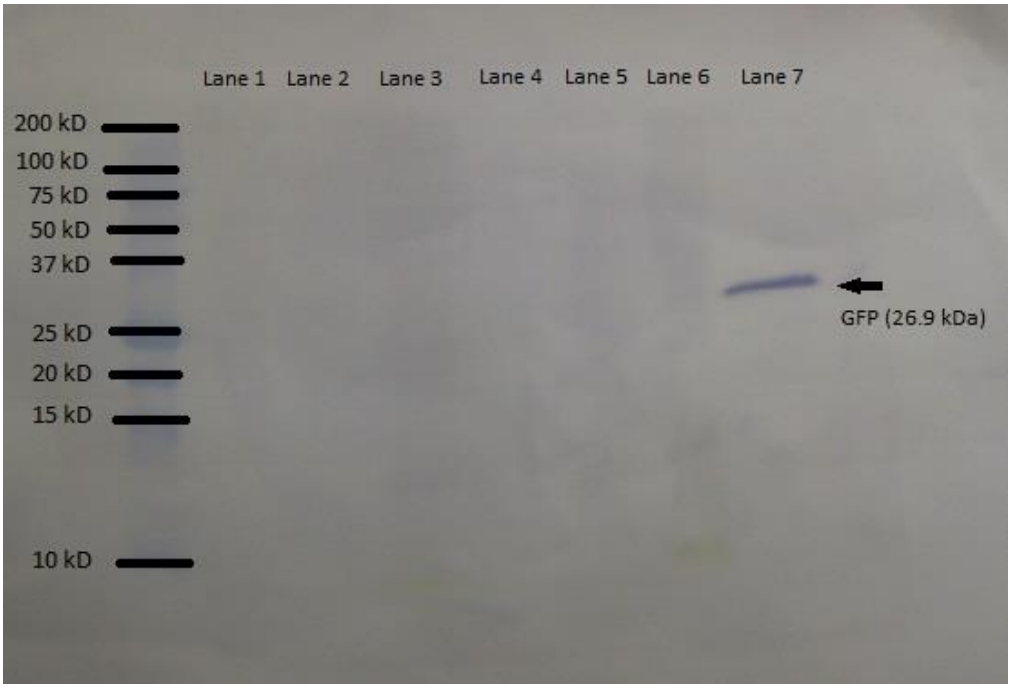

Figure 16: Western blot for non-inoculated plant tissue with control. Lane 1: Stem 1, Lane 2: Root 1, Lane 3: Leaf 1, Lane 4: Stem 2, Lane 5: Root 2, Lane 6: Leaf 2, Lane 7: positive control FSL B2-107 (LM 10403S + GFP) (26.9 kDa).

Total protein extracts from stem, leaf and root sections of two separate, non-inoculated plants were analyzed to ensure no cross reaction of the plant tissue with GFP antibodies (Figure 16). The only lane with a band is that of the control, therefore there is no cross reaction between plant tissue and the GFP antibodies used in the immunohistochemistry experiments.

3.4.4 Immunohistochemistry and microscopy

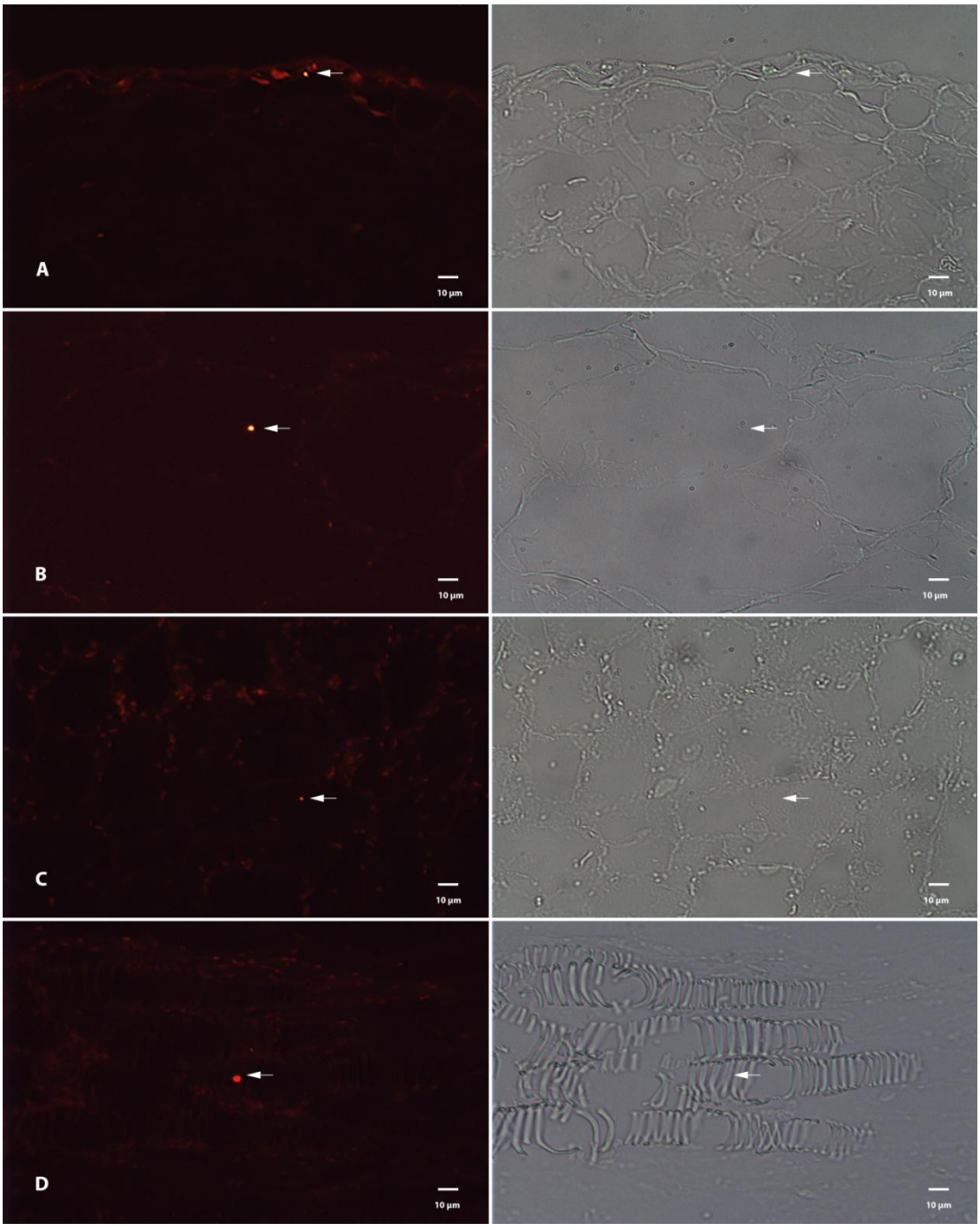

Figure 17: Fluorescence and brightfield paired images of *Listeria monocytogenes* internalized in romaine lettuce tissue. The arrow points to the location of the

bacterium in each set of paired micrographs. The letters represent the following tissue types: A- epidermis, B- cortex, C- pith, D- vascular tissue. Scale bar = 10 µm.

Using immunohistochemical techniques, a total of 539 *L. monocytogenes* bacteria were found to be localized in the tissue of 3 hypocotyl sections of seed contaminated 20 day old romaine lettuce plants. *L. monocytogenes* was found to be associated with every major tissue type including the epidermis (Fig. 17A), cortex (Fig. 17B), pith (Fig. 17C), and vascular tissue (Fig. 17D). The majority of *L. monocytogenes* cells (34.14%) were found to be localized in the pith, which is the innermost part of the plant when viewed as a cross section. Bacteria were also found localized to the epidermis (4.45%), cortex (20.96%), xylem (17.25%), and phloem (8.91%). *L. monocytogenes* cells that were not classified into any of the 5 major tissue types were classified as unknown. This occurred either because a specific tissue type was unidentifiable or tissue type was not adequately differentiated. This often occurred in sections at the beginning or end of the hypocotyls. A total of 14.29% of bacteria were classified as being of unknown tissue type. Based on the statistical analysis conducted, there was no significant variation between plants. Both tissue type and distance to the center of the hypocotyl were found to be significant ($P<0.0001$) as fixed effects in the model. Expected values derived from least square mean estimates also supported bacterial counts being highest in the pith followed by cortex, xylem, phloem and epidermis respectively. Based on pair-wise comparisons of tissue types, all combinations were shown to be

significantly different ($p<0.05$) except for that of epidermis compared to phloem and cortex compared to xylem. Target bacteria were more likely to be found localized closer to the center of the hypocotyl than at the ends.

Table 2: *L. monocytogenes* hypocotyl localization by tissue type

Tissue Type	**Number of *L. monocytogenes* bacteria**	**% of total bacteria**
Epidermis	24	4.45
Cortex	113	20.96
Pith	184	34.14
Xylem	93	17.25
Phloem	48	8.91
Unknown	77	14.29
Total	539	100

In addition to tissue type, the specific apoplastic (extracellular) or symplastic (intracellular) location was recorded for each bacterium. A ratio of 1.07: 1 was observed for apoplastic to symplastic localization.

Assuming each hypocotyl to be a cylinder, the total volume of the 3 hypocotyls was found to be 138.29 mm^3. This results in a bacterial density of about 3.9 bacteria/mm^3.

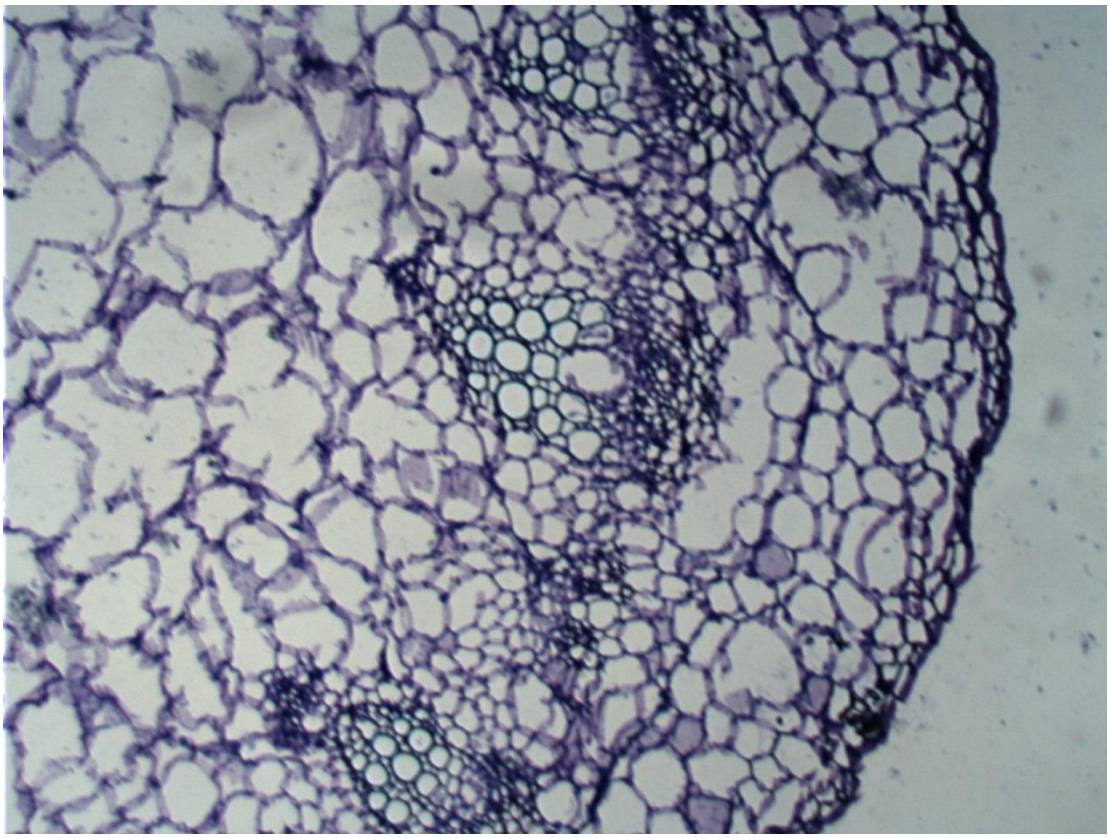

Figure 18: Stained partial cross section of romaine lettuce hypocotyl

The romaine lettuce plant was identified to have 16 vascular bundles via tissue staining with Toluidine blue (Fig. 18). This means that bacteria that are able to enter the vascular bundles are capable of moving throughout the plant since vascular bundles act as a transport mechanism to carry sugars and water throughout the plant system. A total of 26.16% of *L. monocytogenes* bacteria were found in the vascular bundles of romaine lettuce (17.25% in xylem and 8.91% in phloem).

3.5 Discussion

This study effectively demonstrated the ability of *L. monocytogenes* to internalize in the hypocotyl tissue of romaine lettuce. Contaminated seeds that were planted in commercial potting mix, grown in a greenhouse, and harvested after 20 days were used to assess internalization potential. While there are a wide array of methods used to study internalization, a combination of immunohistochemistry and fluorescence microscopy was used in this study. The use of a fluorophore-conjugated antibody allowed for circumventing the direct visualization of GFP following fixation of the tissue and avoided complications as described in the introduction. Additionally, the ability to fix the plant tissue allowed the target bacterium to be cross-linked in place without the potential to be displaced during the other steps required for the dehydration and paraffin embedding steps. Similar internalization analysis methods have been used in other studies, including in one of the pioneering papers in internalization studies (Deering et al., 2012, Itoh et al., 1998).

The results showed that a total of 539 bacteria were identified in the hypocotyl sections resulting in an estimated density of 3.9 bacteria/mm^3. While this may seem like a low density, once internalized, *L. monocytogenes* may have the opportunity to propagate if appropriate conditions are met. It is also most definitely an underestimate of the true number of bacteria present for a variety of reasons. For one, the hypocotyl tissue was sectioned into 10 µm sections. With *L. monocytogenes* being a bacterium that is typically 1 to 1.5 µm in length (Liu, 2006),

multiple bacteria could fit within a single section of tissue if stacked lengthwise. This means that the true number of bacteria could even be as much as six to ten times higher than reported. The numbers reported also only reflect what was found in the hypocotyls and so numbers found throughout the entire plant are likely to be much higher. Additionally, the cells need to be disrupted so as to expose the GFP and allow the antibodies to bind to it. This means that only bacterial cells that have been cut open via the sectioning process will have been exposed and would have permitted antibody binding. Therefore, the number of bacteria found is largely a result of the way the plant tissue was sectioned. More importantly, since the infective dose of *L. monocytogenes* is unknown and largely depends on the health of the individual, the threat of mere pathogen presence to eventually proliferate at a later processing stage and cause illness in at-risk individuals is real. This is especially so for internalized bacteria that most likely would be protected from any kind of cleaning or sanitizing treatment.

Internalized bacteria were found to be associated with every major tissue type which includes the epidermis, cortex, pith, xylem and phloem. The most interesting finding is that of internalized bacteria being found in the xylem. In general, the xylem allows for a continuous route of transport of water and minerals from the roots of the plant through to the leaves. This means that once having gained access to the xylem, *L. monocytogenes* is capable of travelling through it to the rest of the plant where it then has the potential to migrate into other tissue types. When transverse sections were stained with toluidine blue, it was found that romaine

lettuce has 16 vascular bundles which means there is ample opportunity for pathogens to make their way into the plant system vasculature. Other studies have also found evidence of the ability of pathogenic bacteria to be localized within plant vascular bundles. Itoh et al. (1998) found that *E. coli* O157:H7 bacteria were localized in the xylem of the hypocotyl section of radish sprouts that were grown from contaminated seeds. Additionally, Wachtel et al. (2002) also found *E. coli* O157:H7 within the vasculature of the hypocotyl section of leaf lettuce and hypothesized that it must have been in the xylem since it is the only open vessel present. These studies corroborate the fact that pathogens are able to move through the vascular system. While not examined in this study, it is also possible that the pathogen can make its way through the xylem, into the flowers and eventually into the next generation of seeds produced by the plant. The majority of the *L. monocytogenes* cells found were located in the pith (34.14%) which is the innermost region of the plant if looking at a transverse section. Any pathogenic bacteria located in this region will be especially hard to access. Developing any kind of sanitizing treatment to be able to penetrate the plant tissue to such a depth without significantly affecting product quality and/or sensory attributes will prove to be highly challenging.

In typical mammalian host cells, *L. monocytogenes* is considered to be an intracellular pathogen. It was therefore hypothesized that the same mode of invasion might be observed in plant cells and bacteria would be found to localize mostly in the symplast. For this to have been seen in plants, the observed ratio

would have had to have been closer to 1:2 apoplastic to symplastic ratio or higher. The observed ratio of 1.07:1 apoplastic to symplastic localization indicates that the mechanism of internalization in romaine lettuce probably does not follow what occurs in mammalian cells and remains unknown. There are, however, a couple of possible explanations to the seemingly randomized apoplastic or symplastic pathogen localization. For one, bacteria could have been internalized during the growth of the germinating seedling in which uptake of water could have pulled the bacteria in or emerging lateral roots could have allowed for a site of entry. Young seedlings lack developed defense mechanism or essential protective barriers, such as the Casparian strip, to prevent entry of bacteria. Without the Casparian strip, the passive flow of water and solutes is not blocked and bacteria can be pulled into the plant, gain entry to the xylem and potentially be transported through the rest of the developing plant (Warriner et al., 2003). Alternatively*, L. monocytogenes* could have entered through cracks in the seed coat before or during germination and could have spread to the various tissue types by virtue of simply being present at tissue differentiating sites. Regardless of specific method of internalization, the internal tissue of romaine lettuce can provide a protective and nutritious microenvironment for *L. monocytogenes* where it can be protected from external stresses and also from sanitizers.

Internalization of common foodborne pathogens such as *Salmonella* Typhimurium and *E. coli* O157:H7 in produce have been more frequently investigated due to their contributing role in many produce related outbreaks.

Internalization of both abovementioned pathogens has been demonstrated in lettuce (Kroupitski et al., 2009, Solomon et al., 2002b). There have been very few studies, however, that have tested the actual internalization potential of *L. monocytogenes* in pre-harvest produce. Jablasone et al. (2005) did not find any evidence of internalization of *L. monocytogenes* in any of the seedlings of carrot, cress, lettuce or radish plants grown on a solidified hydroponic system. Kutter et al. (2006) designed an experiment in which they exposed barley seedlings to *L. monocytogenes*, *L. innocua*, and *L. ivanovii*. Plants were harvested after 1 to 4 weeks later and found that although the inoculated *Listeria* species colonized the root hair zone, there was no evidence of internalization (Kutter et al., 2006). Millilo et al. (2008) inoculated 21 day old *Arabidopsis thaliana* (thale cress) plants with GFP-expressing *L. monocytogenes* and allowed them to incubate for 24 hours before sampling and analysis by confocal microscopy. The authors found that *L. monocytogenes* was able to internalize in the inner leaf tissue via entry by stomatal openings, but was confined to the extracellular spaces (Milillo et al., 2008). The following two studies, like this study, used lettuce in their experimental design. Standing et al. (2013) found *L. monocytogenes* to be internalized in butterhead lettuce seedlings grown in vermiculite between day 5 and day 14 post- inoculation out of the 28 day experimental period. They also found internalized *L. monocytogenes* in the roots and leaves of hydroponically grown mature butterhead lettuce plants throughout the 4 week monitoring period (Standing et al., 2013). Chitarra et al. (2014) showed that *L. monocytogenes* was found to be internalized in

surface-sterilized leaf tissue analyzed from seedlings that were grown at 24 °C but not 30 °C in substrate inoculated with contaminated irrigation water. A concentration of 3.32 log CFU/g of *L. monocytogenes* was obtained from lettuce leaves at 80 days post-contamination. The authors suggested that the pathogen made its way to the leaf through either the vascular tissue or the apoplast via uptake of contaminated water by the roots (Chitarra et al., 2014). The abovementioned studies were conducted on differing plant types, all with differing native microflora. These factors would have led to different interactions with *L. monocytogenes* and might partially explain why such varied internalization results were obtained.

This study, however, specifically shows confirmation of *L. monocytogenes* internalization in each of the major tissue types within the romaine lettuce hypocotyl, including the plant vasculature. Regardless of the specific pathogen, internalization studies are important because they allow for the understanding of the extent to which a pathogen is able to invade the plant system. It also shows the industry that reliance on surface sanitization may be inadequate and that there is a need for further and improved intervention or sanitization strategies that may be better able to target internalized bacteria. Additionally, the information that a relatively short exposure time of 30 minutes can result in contaminated seeds which then grow into contaminated plants and harbor internalized bacteria is of concern to consumer health.

3.6 List of References

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CHAPTER 4. EXAMINATION OF ROMAINE LETTUCE SEEDS AS A SOURCE OF CONTAMINATION IN RECALLED, BAGGED LETTUCE

4.1 Abstract

Recently, leafy greens have been implicated in an increasing number of recalls from a variety of products due to *L. monocytogenes* contamination. In this study, cultivars of romaine lettuce seeds that were grown by a large commercial producer and that were included in a March 2014 recall were tested as a potential source of *L. monocytogenes* contamination. A total of 100 seeds from each of 16 cultivars of romaine lettuce and 1 cultivar of Radicchio were analyzed for presence of *L. monocytogenes*. The seeds were ground in sterile 0.1 M phosphate buffer and plated on selective Modified Oxford Agar medium. Colonies with *L. monocytogenes* morphology were picked to be amplified using polymerase chain reaction (PCR). The DNA was amplified using 2 primer sets specific to *Listeria* species and *L. monocytogenes*, giving band products of 239 and 706 base pairs respectively. The data showed that 1 out of the 16 cultivars of romaine lettuce tested positive for *L. monocytogenes* contamination. Only a single seed of the River Road cultivar was identified to be contaminated and at a very low level. None of the radicchio seeds analyzed demonstrated presence of *Listeria*. These data indicate that while it is possible that the seed could be a source of potential *L. monocytogenes*

contamination, it is unlikely that the seeds of these cultivars tested were the source in the bagged salad recalls.

4.2 Introduction

A number of outbreaks have been traced back to contaminated seeds. Some have been relatively smaller outbreaks such as the 1997 multistate outbreak of *Escherichia coli* O157:H7 with 85 cases in Virginia and Michigan that was linked to alfalfa sprouts grown from a single lot of contaminated seed produced in Idaho (Breuer *et al.*, 2001). Other outbreaks have been much larger and have had further reaching effects. A 1995 outbreak of *Salmonella enterica* serovar Stanley on alfalfa sprouts involved 242 cases that were not only spread over 17 states domestically, but were also present in Finland. Investigations determined that alfalfa sprout seeds were to blame when it was revealed that the different suppliers in each country utilized the same shipper from the Netherlands for their seeds. How the seeds came to be contaminated, or even the true geographical origin or harvest date of the seeds, was never determined (Mahon et al., 1997). More recently in 2011, nearly 4000 people in 16 countries were affected by a Shiga toxin-producing *E. coli* O104:H4 outbreak centered in Europe. The source of the outbreak was found to be fenugreek sprouts grown from contaminated seeds that were imported from Egypt (Karch et al., 2012). The outbreak resulted in 908 cases of hemolytic uremic syndrome and 50 deaths (CDC, 2013). From the outbreaks discussed above, it is clear that distribution of contaminated seeds can cause widespread and

detrimental consequences due to the current nature of global trade and commerce. Every effort is needed by both the suppliers and distributors of seeds to ensure that potential seed contamination events are minimal throughout the processing, storage and distribution process.

The potential for pathogens on contaminated seeds to persist or internalize once germinated has also been demonstrated. When *Arabidopsis thaliana* seeds were inoculated with 8.3 log CFU of *L. monocytogenes* and sampled after a period of 7 days, it was found that between 4.23 and 4.57 log CFU/cm^2 of the pathogen was recovered (Milillo et al., 2008). Other studies have shown even longer persistence of pathogens on produce grown from contaminated seeds. It was found that *E. coli* O157:H7, *Salmonella enterica* serovar Typhimurium, and *L. monocytogenes* all persisted on the surface of lettuce, radish, cress and spinach grown from respective seeds inoculated with 2 log CFU/ml of each bacteria individually even after a period of 49 days (Jablasone et al., 2005). In agreement with the previous study, *E. coli* P36 (slaughterhouse isolate) was detected even after a period of 42 days on the surface of spinach that was grown from inoculated seeds and planted in soil (Warriner et al., 2003). Only a few other studies have tried to observe the results of produce grown from directly contaminated seeds (Cooley et al., 2003, Habteselassie et al., 2010, Miles et al., 2009). Especially concerning are the studies that have shown the ability of pathogen contamination to carry through to seed production. In other words, bacteria can be passed down through the next generation of plants. Cooley et al. (2003) showed that seeds harvested from inoculated plants were occasionally contaminated themselves. Contaminated seed recovery was

dependent on the method of inoculation of the parent plant, however, indicating that contaminated plants do not always produce contaminated seed and the vertical transmission of pathogens is highly dependent on the interactions of the environment the plant is in. Recovery of contaminated seed from the parent plant was also highly correlated to the presence of contaminated chaff which may indicate cross contamination, but the authors also cited the possibility that seed contamination could have arisen from pathogen invasion of the flower (Cooley et al., 2003). Additionally, another study showed the ability of *E. coli* O157:H7 to invade the inner tissues of Red Delicious apples and attach to both the seed locules and integuments (Burnett et al., 2000). As discussed above, it is evident that contaminated seeds have the potential to produce contaminated plants and that the cycle can continue by contaminated plants producing contaminated seed. Understanding the extent to which this is possible can aid in control measures and reinforce the importance of seed decontamination in the industry. Unfortunately, even seed decontamination is not the perfect solution since pathogenic bacteria could evade sanitizing steps if protected in a surface niche or under the seed coat (Cooley et al., 2003).

It is entirely possible that seeds intended for production are often stored for a period of time, sometimes even a few years. A report by the European Food Safety Authority about the *E. coli* O104:H4 European outbreak stated that the implicated contaminated fenugreek seeds were in fact imported from Egypt into Germany as early as 2009, while another lot was shipped in 2010 (EFSA, 2011). Seeing as how the outbreak occurred in the late spring of 2011, it is clear that the contaminated seeds

were kept in storage for long periods of time and yet the pathogen managed to proliferate on the seeds once planted. While there are very few studies demonstrating long term survival of pathogens on stored seed, Van der Linden at al. (2013) inoculated butterhead lettuce seeds with approximately 8 log CFU/ g of each of two separate *Salmonella enterica* and two *E. coli* O157:H7 strains. Seeds were kept in storage for 2 years before being tested. *Salmonella* was recovered from all seeds tested while *E. coli* was recovered at between 4% to 14% depending on the method of testing used. The stored seeds were also planted and were found to be able to proliferate on the seedlings at concentrations of up to 5.9 log CFU/seedling and 4.4 log CFU/seedling for *Salmonella* and *E. coli* respectively (Van der Linden et al., 2013). Therefore, not only can pathogens survive on seeds for long periods of time, they have also been shown to be able to grow once the seed is planted.

Based on what has been discussed above, it is evident that seeds have the potential to be a source of contamination. While no *L. monocytogenes* outbreaks have been tied to romaine lettuce as yet, there have been many recalls. Between the period of January 2010 to January of 2015, there were roughly 87 recalls involving *L. monocytogenes* contamination in produce. Of these, 18 (~20%) were specific to romaine lettuce or romaine lettuce containing products (FDA, 2015). In March 2014, a large precautionary recall was put in effect for 4 varieties of bagged salad mixes that were distributed in 15 different states by a large, commercial producer (FDA, 2014). The contaminated blend which prompted the recall included romaine lettuce and radicchio.

It is hypothesized that romaine lettuce seeds could be a potential source of *L. monocytogenes* contamination and may have contributed to recalls in product that has been grown and harvested from them. The objective of the study was to identify whether romaine lettuce seeds have *L. monocytogenes* contamination via testing through PCR and gel electrophoresis.

4.3 Materials and Methods

4.3.1 Polymerase Chain Reaction (PCR) on romaine lettuce seeds

Sixteen different cultivars of romaine lettuce and one cultivar of radicchio were obtained from a large commercial grower with some of the cultivars grown implicated in recalls. From each cultivar, 100 seeds were individually soaked overnight in sterile 1 ml of 0.1 M phosphate buffer in an Eppendorf tube. Each seed was then ground using a sterile mini pestle, vortexed, and 100 µl of the homogenate was plated on Modified Oxford Agar for each of a 10^{-1} to 10^{-4} dilution series. Plates were then incubated at 30°C for 48 hours. Isolated colonies with distinctive *L. monocytogenes* morphology were individually picked and each placed into 100 µl of sterile water in an Eppendorf tube before being microwaved for 3 minutes at 1 minute intervals to create dirty lysates. A representative sample of any other esculin positive colonies was also collected to eliminate any subjective bias in colony picking. Dirty lysates were briefly spun in a centrifuge and the supernatant was used in PCR analysis. Each PCR amplification was

conducted in a total volume of 20 µl with primers designed by Hudson et. al (2001). Primers 310F (5'-GCCTGCAAGTCCTAAGACGCCAATC) and 1016R (5'-CTTGCAACTGCTCTTTAGTAACAGC) were used to check for amplification of the Listeriolysin O encoding gene of *L. monocytogenes*, producing a 706 bp band while primers L318F (5'-GGGGAACCCACTATCTTTAGTC) and L559R (5'-GGGCCTTTCCAGACCGCTTCA) were used to check for amplification of the 23S rRNA gene of *Listeria* species, producing a 239 bp band. Amplification parameters were as follows: denaturation at 95 °C for 1 minute, annealing at 57 °C for 1 minute and extension at 72 °C for 1 minute for a 40 cycle repetition. The final extension step ran for 8 minutes at 72 °C. PCR products were analyzed via gel electrophoresis using a 2% gel (45 ml dH_2O, 5 ml 10x TBE (0.89 mol l^{-1} Tris base, 0.02 mol l^{-1} EDTA, 0.89 mol l^{-1} boric acid), 1g agarose) and run in 1X TBE buffer for 45 minutes at 120V. Gels were visualized using Gel Doc XR+ imaging system (Bio-Rad Laboratories, Hercules, CA).

4.4 Results

4.4.1 PCR on romaine lettuce seeds

Of the 16 possible varieties of romaine lettuce and 1 cultivar of radicchio tested for a total for 1700 seeds, only one seed from one romaine lettuce cultivar, River Road, was found to be contaminated. The 10^{-1} plated dilution of the single contaminated seed

yielded only 3 *L. monocytogenes* colonies. The cultivar of radicchio seed used in the recalled product did not show any contamination.

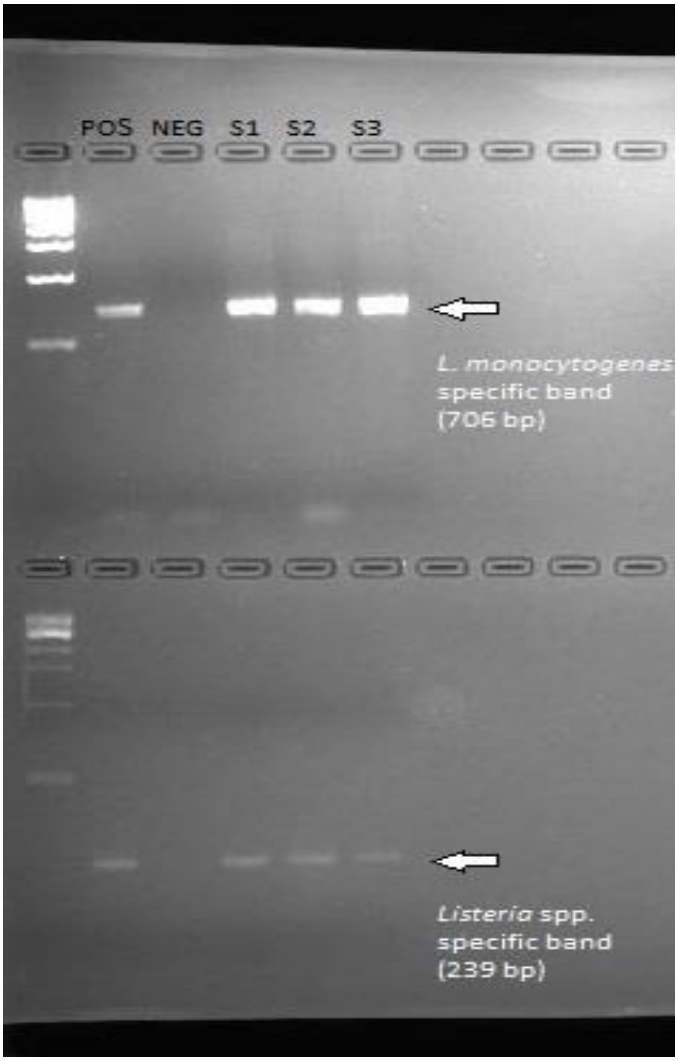

Figure 19: Gel electrophoresis image of *L. monocytogenes* colonies from River Road seed. The first three lanes are that of the ladder, positive control, and negative control respectively. Lanes labelled S1, S2, S3 were the only 3 confirmed *L. monocytogenes* colonies from the River Road cultivar seed homogenate that was plated.

As labelled on Fig. 19 above, the 3 samples that were confirmed to be *L. monocytogenes* showed correct band sizes of 706 bp for the *L. monocytogenes* specific primers and 239 bp for the *Listeria* spp. specific primers.

4.5 Discussion

Seeds from 16 possible varieties of romaine lettuce and 1 cultivar of radicchio from the commercial grower that match the production period of the recalled product were tested. Of these, only one seed from one romaine lettuce cultivar, River Road, was found to be contaminated. The results indicate that contamination was present at a very low level. Overall, only 1 of the 1700 total seeds tested showed any indication of *L. monocytogenes* contamination. Due to the lack of significant contamination findings on the sample of seeds tested, the recalled product was unlikely to have been contaminated prior to planting. However, it does not completely rule the seeds out as a source. The sample size of 100 seeds per cultivar is rather small compared to the total number of seeds that would be present in a given lot of a specific cultivar. Contamination of seeds is often not evenly distributed in a seed lot and there is a possibility that these can go undetected due to the inability to test the entire lot (Breuer et al., 2001, Mahon et al., 1997). Additionally, as shown by the previous studies, contaminated seed can result in contaminated plants and with germination conditions being able to support the proliferation of bacteria, the initial contamination load may not even have to be that high.

Effects from contaminated seeds can be far-reaching since single lots of seeds are often distributed to many growers who then distribute to various retailers. Risk reduction is crucial to all steps in the farm to fork continuum of the produce process, including the pre-production stage. Checks such as seed disinfection and testing should continue to be used in the industry to prevent increased risk for the consumer. Seed disinfection may not allow for the complete elimination of pathogens, because these may reside in cracks or uneven surfaces of the seed coat, but treatments do aid in lowering bacterial populations to an extent (Taormina et al., 1999). Additionally, the storage of seeds under appropriate conditions which prevent bacterial proliferation, but that do not affect seed quality, is necessary. Following this line of reasoning, mixing of seed lots may allow for an increased chance of cross contamination and should be prevented as much as possible.

There continues to be difficulties with identification and traceback of contaminated seeds in the industry. A further effort into studying the long term survival of pathogens on seeds, as well as elucidating their cross contamination potential is needed. Future work relating to this study could involve germinating the seeds of the various cultivars and testing the seedlings for presence of *L. monocytogenes* in order to ascertain whether germination conditions can increase the likelihood of finding the pathogen.

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CONCLUSIONS

The overall purpose of this thesis project was to determine interactions of *L. monocytogenes* with romaine lettuce under pre-harvest conditions. The first objective was to understand if and how *L. monocytogenes* persisted on romaine lettuce over a period of time and how this persistence was influenced by various factors such as *L. monocytogenes* strain, lettuce cultivars or growth media. Results showed that under sterile conditions, romaine lettuce plants were able to support the growth of *L. monocytogenes* at relatively high levels of 4.4 to 7.8 log CFU/g after a 60 day period. While sterile conditions obviously do not mimic what occurs under open field conditions such as those used during lettuce production, this data could be used to examine how *L. monocytogenes* would persist under more ideal conditions and provide a worst case scenario for pathogen persistence. When germinated in more traditional forms of plant growth media, *L. monocytogenes* persistence did not maintain at detectable levels (2 log CFU/g) for as long as compared to the sterile agar trials. The more rapid decline in persistence is likely due to the increased presence of competing soil microflora in the commercial potting mix and 75% Indiana top soil trials. Based on the results of this study, *L. monocytogenes* is unlikely to be able to persist until the harvest period of romaine lettuce plants grown in field production sites. Additionally, neither *L. monocytogenes*

strain differences nor the presence of a clay coating on the seed were found to significantly affect persistence. Cultivar differences, however, were found to have the potential to influence pathogen persistence. This information is useful to growers since being able to mitigate use of cultivars which support persistence of *L. monocytogenes* would aid in reducing food safety risk. Further work regarding such studies could perhaps look into whether actual produce isolates of *L. monocytogenes* would differ in their persistence capabilities. Additionally, more types of soil need to be examined with the produce and pathogen combination of romaine lettuce and *L. monocytogenes,* especially in geographical areas where romaine production is heaviest. Another interesting area of future work would be to test how persistence levels are altered with various other inoculation levels of *L. monocytogenes* on romaine lettuce seeds and whether there is a minimum pathogen load required for the establishment of detectable persistence.

The second objective was to determine whether *L. monocytogenes* was able to internalize within the romaine lettuce tissue, as the ability to do so could contribute to the success of its persistence. This was confirmed as a result of this study via the use of GFP tagged *L. monocytogenes* and standard immunohistochemistry techniques. A total of 539 *L. monocytogenes* cells were found in the hypocotyls of 3 plant replicates with majority localizing in the pith, followed by the cortex, xylem, phloem and epidermis respectively. The presence of the pathogen in the innermost portion of the plant stem could pose a hurdle towards lettuce sanitization efforts and provide a protected environment for proliferation. Additionally, the presence of *L. monocytogenes* in the

plant vasculature indicates the potential for the pathogen to be able to travel within and throughout the plant system. Since *L. monocytogenes* is ubiquitous in natural environments like soil, any of the bacteria that manage to internalize via the root and gain access to the transport system could eventually end up in edible tissue. The results also showed that the mechanism of internalization in romaine lettuce probably does not follow what occurs in mammalian cells and remains unknown. Elucidating the mechanism of internalization, perhaps via genomic experiments which look at how gene regulation changes when *L. monocytogenes* is associated with romaine, is an important future step. Furthermore, studying if internalization is still detectable closer to harvest period or even how the level of *L. monocytogenes* internalization varies in different lettuce types will help contribute to a better understanding of the pathogen-plant interaction and possibly even aid in developing prevention strategies.

The final study of this work involved examining whether romaine lettuce seeds could have been the source of *L. monocytogenes* contamination in a commercial bagged salad recall. In this particular instance, the recalled product was unlikely to have been contaminated prior to planting due to the lack of significant contamination findings on the sample of seeds tested. There is, however, the potential for such a scenario to happen as evidenced by the ability of contaminated seeds to grow into contaminated plants in the studies discussed above. Continued efforts are required into the proper collection, storage, and delivery of seeds. This is especially so with the increasingly global nature of food production and the various additional points of possible contamination included in the distribution process.