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THE MICROBIAL ECOLOGY OF LISTERIA MONOCYTOGENES AS IMPACTED BY THREE ENVIRONMENTS: A CHEESE MICROBIAL COMMUNITY; A FARM ENVIRONMENT; AND A SOIL MICROBIAL COMMUNITY

A Dissertation Presented

by

Panagiotis Lekkas

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Abstract

The aim of the first study was to investigate the effects of *L. monocytogenes* on the composition of the surface microflora on washed rind soft cheese. Two trials with washed rind cheeses that were inoculated with 100 cfu cm$^{-2}$ of a *L. monocytogenes* six strain cocktail were conducted. Surface cheese rind samples were collected from both control and inoculated cheeses every 7 days. Cheese rind samples were analyzed through the standard BAM method for enumeration of *L. monocytogenes* and through amplification of the V4 region of 16S rRNA and ITS regions for identification of the surface rind bacterial and fungal communities, respectively. Our data showed that *Pseudomonas* spp. significantly changed the composition of the microorganisms on the surface of the rind while *L. monocytogenes* had little effect. Although the concentration of *L. monocytogenes* increased to levels of 10$^6$ cfu cm$^{-2}$ based on the enumeration data, the genetic data was not able to identify it in the flora, which is a limitation of molecular methods used for identification of pathogens in foods.

For the second study the presence and incidence of *L. monocytogenes* on farms that either produce raw milk cheese or supply the milk for raw milk cheese production was investigated. Five farms were visited and in total 266 samples were collected from barn, environmental, and milk sites. *L. monocytogenes* prevalence was found to be at 6% from all the farms tested. Samples were identified to the genus level through a modified BAM method and speciated though multiplex PCR. Included in the pathogenic isolates was a DUP-1042B *L. monocytogenes* strain that has been implicated in major outbreaks. Results from this study continue to support the fact that contaminated silage can be an important reservoir of the pathogen in a dairy farm setting. From our data we identified that drinking water sources for the animals are also an important reservoir of *L. monocytogenes* in farm environments.

Lastly manure amended soils in the northeastern U.S. were tested for the presence and survival of rifampicin resistant *Escherichia coli* (*rE. coli*), generic *E. coli* (*gE. coli*) and *Listeria* spp. Both *gE. coli* and *rE. coli* samples were processed using either direct enumeration, MPN or bag enrichment methods. Samples were taken from both tilled and surface dairy solid manure-amended plots. *Listeria* samples were processed using a modified BAM method. *Listeria* presence was constant throughout the study. In contrast, *rE. coli* and *gE. coli* levels declined with time. The main conclusions of this study were that soil type, location and physical characteristics have a significant role in the survival of bacterial populations of *rE. coli*, *gE. coli* and *Listeria* spp. in soil. Tilling of soils results in increased survival of the bacterial population due to the fact that it increases soil pore size and facilitates moisture entry. Data from this research will assist in the creation of preventative measures that lead to the elimination of pathogen reservoirs. It will be further used to verify that a 120 day interval following manure application should be sufficient to ensure food safety of edible crops subsequently planted on these soils.
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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

This dissertation examined the microbial ecology of *Listeria monocytogenes* as impacted by three environments: a cheese microbial community; a farm environment; and a soil microbial community. Most human foodborne infections are usually associated with a high illness incidence balanced by a low morbidity and mortality. Human listeriosis, which is a rare but potentially very severe infection, is associated with a mortality rate of up to 30% (Graves et al., 1994; Kyoui et al., 2014; Murphy et al., 2007; Back et al., 1993; West, 2008) even when antimicrobial treatment is administered (Lecuit et al., 2007). This results in a high human and economic loss, despite its relative low incidence (Lecuit et al., 2007). A comprehensive study, looking at illnesses and deaths from foodborne pathogens in the U.S. during 2000-2008, (Scallan et al., 2011), estimated that 19% of deaths were caused by *L. monocytogenes* with a 94% hospitalization rate. The authors concluded that most deaths due to foodborne illness in the U.S. were caused by nontyphoidal *Salmonella* spp. (378), *L. monocytogenes* (255) and norovirus (149). A review of *L. monocytogenes* and its characteristics will be presented, along with a discussion of where it is found, and how it is isolated. Methods of subtyping used to discriminate species and strains will be reviewed, followed by a discussion of important outbreaks and factors which led to these outbreaks. The thesis will end with a discussion of strategies to bring about control of *Listeria* in food processing and farm environments.
1.2 *Listeria* - Background.

Members of the genus *Listeria* are Gram positive (Swaminathan and Gerner-Smidt, 2007; Wing et al., 2002), rod-shaped (bacillus) bacteria that belong to the *Firmicutes* phylum (Conly and Johnston, 2008; Cossart, 2011). They are facultatively anaerobic, non-spore forming, approximately 0.5 µm in width and 1-1.5µm in length, with a low G + C (38%) content (Liu, 2006; Wong, 2004; Cossart, 2007; Vázquez-Boland et al., 2001a; Cossart, 2011) and belong taxonomically to the *Clostridium* sub branch (Allerberger et al., 2003). *Listeria* species are common in the environment (Haley et al., 2015; Schwartzman et al., 2011; Fang et al., 2013) and can grow over a wide range of pH extremes (4.3 – 9.6), temperatures (1 – 45°C) and salt concentrations (up to 10 %). This ability to survive and multiply under conditions frequently used for food preservation makes *Listeria* particularly problematic to the food industry (Roberts and Weidmann, 2003; Borucki and Call, 2003; Fang et al., 2013).

The genus *Listeria* includes ten species: *L. monocytogenes*, *L. innocua*, *L. welshmeri*, *L. seeligeri*, *L. ivanovii*, *L. grayi*, *L. marthii*, *L. rocourtiae*, *L. weihenstephanensis* and *L. fleischmannii* (Graves et al., 2010; den Bakker et al., 2014). More recently den Bakker et al., (2014) isolated 18 *Listeria* like isolates from two US states (Colorado and Florida) producing 5 new species: *L. floridensis*, *L. aquatica*, *L. cornellensis*, *L. grandensis* and *L. riparia*, all of which were non-pathogenic. Of the species of *Listeria* identified thus far, only *L. monocytogenes* is pathogenic to humans and animals, and *L. ivanovii* has been shown to be pathogenic to animals, predominantly in ruminants and has rarely been implicated with human infections (Orsi et al., 2011; Cossart,
Gouin et al., 1994; Roberts and Weidmann, 2003; den Bakker et al., 2010; McLauchlin, 1997). L. seeligeri, although it contains gene homologues of the virulence cluster, has rarely been linked to a human or animal outbreaks (Graves et al., 2010). L. innocua, the most closely related species to L. monocytogenes, strain EGD-e (1/2a serotype) was the first genome sequence published by Glaser et al., (2001). Comparison among L. monocytogenes and L. innocua genomes showed that 15% of the genes are absent in the latter species and in particular a 10-kb virulence locus which is present in L. ivanovii (Cossart, 2011). Investigating the genome evolution of the eight (at that time) recognized species (L. monocytogenes, L. innocua, L. welshmeri, L. seeligeri, L. ivanovii, L. grayi, L. marthii and L. rocourtiae) of the genus Listeria, den Bakker and others (2010) concluded that the genus evolves through the loss of virulence rather than acquisition of virulence characteristics. Through whole genome sequencing (WGS) den Bakker et al., (2014) were able to differentiate Listeria spp. into 3 clades: clade (i) represented by L. monocytogenes, L. innocua, L. welshmeri, L. seeligeri, L. ivanovii and L. marthi; clade (ii) consists of L. fleischmannii, L. floridensis, and L. aquatic; and clade (iii) includes L. rocourtiae, L. weihenstephanensis, L. cornellensis, L. grandensis and L. riparia.

The majority of Listeria spp. has been separated through subtyping methods into two major lineages, I and II (Orsi et al., 2011), which were first identified in 1989. Later two more lineages, III and IV, were added. Lineage I is represented by serotypes 1/2b, 3a, 3b, 3c and 4b. Lineage II consists of serotypes 1/2a, 1/2c, 3b, 4b, 4e, and 4d. Lineage III and IV were created for serotypes 4c, 4a and atypical 4b (Cossart, 2011; Orsi et al., 2011; Nightingale et al., 2006; Nadon et al., 2001).
1.3 *Listeria monocytogenes*

The bacterium *L. monocytogenes* was first recognized as a cause of infection in laboratory animals at Cambridge University by Murray et al., (1924) and was initially named *Bacterium monocytogenes* (Conly and Johnston, 2008; McLauchlin et al., 2004; Cossart, 2007; Cossart, 2011). Pirie (1927) also isolated the same bacterium from gerbils in South Africa and named it *Listeria hepatolytica*. Later in 1940 he suggested that the name be changed to *L. monocytogenes* (McCarthy, 1990). It was first thought to be an animal pathogen, but it was later identified to also be infectious to humans (McLauchlin et al., 2004). Most of the human infections are transmitted through food (Carpentier and Cerf, 2011). *L. monocytogenes* strains are serotyped based on the variation in the somatic (O) and flagellar (H) antigens (Borucki and Call, 2003; Liu, 2006). Despite the fact that there are 13 different serotypes currently identified, 98% of the confirmed human listeriosis cases belong to the serotypes 1/2a, 1/2b and 4b (Orsi et al., 2011; Liu, 2006). The most common serotype isolated from food is 1/2a, however the serotype that causes the majority of the foodborne outbreaks is 4b (Gilot et al., 1996; Borucki and Call, 2003; Liu, 2006; Kathariou, 2002). In sheep serotypes 1/2b and 4b cause encephalitis while serotype 1/2a is associated with septicemia and abortions cases (Liu, 2006). *L. monocytogenes* is psychrotrophic and is able to multiply at temperatures below 0°C, between pH values of 4.6 to 9.5, under high salt conditions and at water activity as low as 0.92 (Carpentier and Cerf, 2011; Cataldo et al., 2007; Swaminathan and Gerner-Smidt, 2007). *L. monocytogenes* can find favorable conditions for growth on floors, drains, and food industry equipment including the cold wet atmosphere of refrigerated rooms (Carpentier and Cerf, 2011). The
unique ability of *L. monocytogenes* to survive in a broad range of ecosystems is associated with its large number of regulatory genes and proteins (approximately 11.6% of all predicted genes); 16 two-component regulatory systems; and the largest phosphotransferase system described in bacteria to this date (Hamon et al., 2006). Initially *L. monocytogenes* was recognized as a human pathogen that caused sporadic human infections and was mainly associated with workers coming into contact with diseased animals (Conly and Johnston, 2008). In the 1980’s, after several outbreaks including Vacherin Mont d’Or in Switzerland in 1983-1987; improperly pasteurized milk in the U.S. in 1983; Mexican style cheese in the U.S. in 1995; that interest for the pathogen among food manufacturers started to emerge (Lecuit, 2007; Fleming et al., 1985; Pintado et al., 2005; Pritchard et al., 1995). Since then, *L. monocytogenes* outbreaks have been associated with the consumption of contaminated foods which include dairy products, poultry, pork, hot dogs, fish, vegetables and seafood (Donnelly, 2001; Petruzelli et al., 2010; Conly and Johnson, 2008; Gould et al., 2014; Guillier et al., 2008; Koch, 2010).

1.4 Pathogenicity and virulence

Following ingestion of contaminated food, *L. monocytogenes* is able to cross the intestinal barrier, after it has withstood exposure to high acidity, bile salts, non-specific inflammatory attacks and proteolytic enzymes (Jeyaletchumi et al., 2010), whereby it disseminates from the mesenteric lymph nodes to the spleen and the liver. *L. monocytogenes* has the ability to breach endothelial and epithelial barriers (Lecuit, 2007; Hain et al., 2006; Vázquez-Boland et al., 2001a). The range of cells that this pathogen can survive within includes non-phagocytic and phagocytic cells. The latter is especially
important since phagocytes possess mechanisms that are used to destroy ingested bacteria. The areas where the pathogen can reside after entry to the host cell are within an acidic and hydrolytically competent phagolysosomal vacuole, inside a vacuole that has not fused with a lysosome, and in the host cell cytosol. (Wilson et al., 2002).

*L. monocytogenes* and *L. ivanovii* are facultative intracellular parasites that are able to replicate and spread from cell to cell using an actin motility process (Hain et al., 2006, Cossart, 2011, Jeyaletchumi et al., 2010). Initial symptoms of listeriosis may include digestive symptoms such as nausea, abdominal pain, aqueous diarrhea and fever (Lecuit, 2007). If not controlled properly, *L. monocytogenes* infection can cause prolonged and asymptomatic bacteremia. *L. monocytogenes* can cross the blood-brain barrier and placenta, and cause infection, resulting in meningitis or encephalitis, abortions in pregnant women, infections in neonates, and death. Additional high risk groups include immunocompromised individuals such as cancer patients or those undergoing treatment with steroids or cytotoxic drugs and elderly individuals. (Lecuit, 2007; Jadhav et al., 2012; Hamon, 2006; Kousta et al., 2010; Vivant et al., 2013; Koch et al., 2010. Donnelly, 2001). Apart from predisposing health conditions, age may also be a determining factor in contracting listeriosis based on the fact that people over 60 years of age have a case-fatality rate of 63% while for people <40 years of age the rate drops to 11% (Donnelly, 2001).

*Listeria* spp. has a characteristic intracellular life cycle with the following steps: (i) entry into the host cell, (ii) early escape from the phagocytic vacuole, (iii) multiplication in the host cell cytoplasm, (iv) directional intra-cytosolic motility by induction of actin polymerization, (v) protrusion of centrifugally moving bacteria within cytoplasmic
evaginations, (vi) escape of secondary vacuole, and (vii) cycle re-initiation (Vázquez-Boland et al., 2001b, Silva et al. 2013). Infection of macrophages by \textit{L. monocytogenes} is a passive process, but entry to the non-phagocytic cells is dependent on a series of bacterial surface proteins and other virulence factors (Hamon et al., 2006).

1.5 Internalins

There are two subfamilies of internalins. The first subfamily consists of large proteins (70–80 kDa), such as InlA and InlB, which are attached via their C-terminal regions to the bacterial cell wall. Other internalins belonging to this group include inlC, inlD, inlE, inlF, inlG and inlH, all found in \textit{L. monocytogenes}. The second group includes proteins generally much smaller in size (25–30 kDa), which lack the C-terminal cell-wall anchor region and are released in the extracellular medium such as InlC (or IrpA) present in \textit{L. monocytogenes} and i-InlC, i-InlD, i-InlE, i-InlF and i-InlG all of which have been identified in \textit{L. ivanovii} (Vázquez-Boland et al., 2001b).

Entry of \textit{L. monocytogenes} in the host cell and incorporation into a membrane bound vacuole is facilitated by two proteins: Internalin A and B (InlA, InlB) (Hain et al., 2006; Cossart, 2011; Jeyaletchumi et al. 2010), both of which are encoded by \textit{inlAB} operon (Chaturongakul et al., 2008; Pan et al., 2014). Both InlA and InlB are necessary and sufficient for bacterial entry into cell types such as enterocytes, hepatocytes, fibroblasts, epithelial cells, and endothelial cells (Unnerstad et al., 2001; Hamon et al., 2006, de las Heras et al., 2011). InlA exhibits high specificity for human E-cadherin while InlB binds the cellular receptor Met, a tyrosine kinase protein, which is also the endogenous ligand of the hepatocyte growth factor (HGF) (Hain et al., 2006; Hamon et al., 2006; Cossart, 2009).
InlA promotes local cytoskeletal rearrangements in the host cell to stimulate uptake of *L. monocytogenes* by epithelial cells (Hamon et al., 2006). InlA species specificity during infection by *L. monocytogenes* was investigated by Lecuit et al., (2001) using a transgenic mouse model that produced human E-cadherin. Murine E-cadherin is not well recognized by InlA (Cossart, 2007) and as a result there was very low rate of infection occurring in these models through oral ingestion of *L. monocytogenes*. Prior to the construction of the transgenic mouse, the guinea pig was the animal model used to study the interaction and significance of the inlA-E-cadherin complex (Lecuit, 2007). Covalent linkage of InlA to the extracellular domain of human E-cadherin causes the intracellular domain to form a complex with the cytoskeleton through catenins, which in turn rearranges the cytoskeleton and allow the entry of *L. monocytogenes* to the host cells (Hamon et al., 2006).

The first role of InlB, which is similar to InlA, is the rearrangement of cytoskeletal structure to facilitate the entry of *L. monocytogenes* in the host cell (Hamon et al., 2006). InlB assists entry into a wider range of cell types (epithelial cells, fibroblasts, hepatocytes and endothelial cells) from different animal species (Vázquez-Boland et al., 2001a). Binding of the InlB activates the protein-tyrosine-kinase activity of Met as well as the phosphatidylinositol 3-kinase (PI3K) and the Ras-mitogen-activated protein kinase (MARK) pathway, all of which are required for the uptake process. These pathways are also activated by the binding of the hepatocyte growth factor (HGF) to Met, which is important for cellular survival and proliferation signals. However InlB seems to induce a more potent activation than HGF. This result can be explained by the fact that InlB binds the globular part of complement component C1q (gClqR), which can act as a co-receptor.
for InlB (Hamon et al., 2006; Hain et al., 2006). InlB’s secondary role is to act as a protein signaling molecule (Cossart, 2011).

A third internalin, Inl C, is produced after the bacterium has entered the host cell and its function is to interact with IκB kinase (IKKα) which in turn prevents activation of nuclear factor-κB (NF-κB), a proinflammatory pathway (Lawrence, 2009) and as a result dampens the hosts innate responses (Cossart, 2011). InlC which is regulated by prfA, is also interacting with the actin binding protein Tuba, which regulates the passage from one cell to another (Cossart, 2011). InlJ, Vip, and a GW protein (Auto) were also discovered, through genome comparison of L. monocytogenes and L. innocua, as three other proteins that assist L. monocytogenes entry to the host cell. The function of InlJ is not yet know however Vip is thought to interact with the signaling molecule for Toll-like receptor, gp96, and GW interacts with components of the innate immune system (Hamon et al., 2006).

1.6 Listeria pathogenicity island -1 (LIPI-1)

L. monocytogenes has a versatile arsenal of virulence factors that have been well identified and characterized at the molecular level (Vázquez-Boland et al., 2001b). Listeria pathogenicity island (LIPI-1) contains 6 virulence factors flanked by phosphoribosyl PPI synthetase gene (prs) and lactate dehydrogenase gene (ldh) (Gouin et al., 1994; Hain et al., 2006) that are necessary for its intracellular lifestyle. These are: prfA (pleiotropic virulence transcriptional regulator), plcA, plcB (both encoding phospholipases), hly (listeriolysin O), mpl (metalloprotease) and actA (involved in actin-mediated motility) (Gilmour et al., 2010; Cossart, 2011; Jeyaletchumi et al., 2010; Vázquez-Boland et al., 2001a). These virulence
genes are physically linked in a 9-kb chromosomal island (Vázquez-Boland et al., 2001b; Hain et al., 2006).

The success of *L. monocytogenes* as a pathogen depends on virulence factors that are regulated by PrfA and σ^B^ (one of five sigma factors in *L. monocytogenes*). InlA, InlB, and InlC, hyl, (which encodes for LLO), actin polymerization factor ActA, metalloprotease (Mpl), phospholipases A and B (PlcA, PlcB), and the sugar uptake system Uhp T, which are all controlled by PrfA (Cossart, 2011; Jeyaletchumi et al., 2010; Vázquez-Boland et al., 2001b). PrfA is a transcriptional factor that belongs to the cAMP receptor protein family (Crp/Cap-Fnr family) (de las Heras et al., 2011; Cossart, 2011; Scortti et al., 2007). The structure of this protein is a 235-residue, 27 kDa, symmetric homodimer that is characteristic of proteins of this family (de las Heras et al., 2011; Scortti et al., 2007). Except from the PrfA regulon, which is tightly controlled by PrfA, there are 145 other *L. monocytogenes* genes that are influenced by PrfA (Scortti et al., 2007; de las Heras et al., 2011). Regulation of PrfA protein is achieved by a 5’-UTR thermosensor (Cossart, 2011; de las Heras et al., 2011). During exponential growth *prfA* is mainly transcribed as a bicistronic mRNA for the plcA promoter. At low temperatures of ≤ 30°C, a secondary structure, a RNA hair pin, is formed that masks the ribosome binding site. At 37°C the structure partially melts and exposes the ribosome binding site that allows translation to occur (Hamon et al., 2006; de las Heras et al., 2011; Loh et al., 2009). Transcriptional control of PrfA is also dependent on the PrfA concentration in the bacterial cytosol (Scortii et al., 2007). Monocistronic synthesis of PrfA occurs during stationary phase and it is driven by two promoters, P1*prfA* and P2*prfA* located upstream from the *prfA* gene. In
contrast a bicistronic mRNA synthesis that carries the message for both *plcA* and *prfA* occurs during exponential growth (Hamon et al., 2006; Scortti et al, 2007). P1prfA and P2prfA are responsible for maintaining a basal level of transcription of PrfA and are controlled by sigma factors. Vegetative sigma factor σA regulates P1prfA while P2prfA is controlled by both sigma factors σA and σB (Hamon et al., 2006; de las Heras et al., 2011; Nadon et al., 2002; Chaturongakul et al., 2008). It was recently discovered that certain 5’-UTRs, termed riboswitches, inhibit translation by acting as noncoding RNAs (ncRNA) and interacting with the 5’-UTR of the mRNA that encodes PrfA and thus providing an additional level of control for this important virulence factor (Loh et al., 2009).

Apart from LIPI-1, PrfA regulates additional virulence determinants, such as *bsh* encoding a bile salt hydrolase essential for survival within the gut, and *uhpT* encoding for a hexose phosphate permease for utilization of host carbon sources (Hain et al., 2006; Gilmour et al., 2010).

The alternative σ factor σB is encoded by the *sigB* gene in *L. monocytogenes* (Moorhead and Dykes, 2003). Alternative sigma factors have been demonstrated to contribute to cellular survival under stressful conditions. For example, the stress-responsive alternative sigma factor σB contributes to the ability of stationary-phase *L. monocytogenes* cells to adapt to and resume growth at reduced temperatures, and has also been shown to contribute to *L. monocytogenes* survival under in vitro conditions of oxidative stress, starvation, bile salts, and reduced pH (Chaturongakul et al., 2008; Nadon et al., 2002).
The *hly* gene has a central location and is responsible for producing listeriolysin O (LLO) (Cossart, 2011; Jeyaletchumi et al., 2010). LLO is required for disruption of the phagocytic vacuole and the release of bacteria in the cytoplasm, a prerequisite for intracellular proliferation (Vázquez-Boland et al., 2001b). Escaping the vacuole is achieved by activation of the LLO gene in animal models but not in humans. In humans *plcA* and *plcB* are required for escaping the vacuole with the latter being essential for the lysis of the secondary vacuole (Cossart, 2011). LLO is highly affected by the environmental pH with higher levels of expression observed under acidic pH levels (pH<6) and lower activity levels observed at neutral pH (Hamon et al., 2006; Cossart, 2011).

Listeriolysin S (LLS) is another virulence factor that was identified by Cotter et al., (2008). This secondary hemolysin is strain specific and is only found (to date) on lineage I strains. This second hemolysin is only induced under oxidative stress conditions, contributes to virulence of the pathogen as assessed by murine and human polymorphonuclear neutrophil–based studies, and is similar to the peptide streptolysin S produced by *Streptococcus* (Cotter et al., 2008; Cossart, 2011).

The surface protein *ActA* is responsible for the recruitment of the Arp2/3 complex, promotes actin polymerization, and formation of the actin tail required for movement both inter and intracellularly. It also protects bacteria from autophagy (Cossart, 2011; Vázquez-Boland et al., 2001b).

Recent research has shown that there are cytosolic compounds that the bacterial pathogens have adapted to and are using to promote virulence factors through allosteric binding. One such example is the glutathione. Reniere and others (2015) concluded in their
study that glutathione from both bacterial and host-synthesized sources contribute to the expression of the prfA virulence regulator of L. monocytogenes. Glutathione is present in the cytosol of all host cells, therefore intracellular pathogens have adapted to import it; such is the case for L. monocytogenes and for other pathogens, like Francisella tularensis. The results from this study suggest that this compound is used as a switch for the bacteria to change between the saprophytic to the pathogenic life cycle and vice versa. Reniere et al., 2015 concluded that high levels of glutathione will promote the change to the pathogenic lifecycle.

The single gene fri that encodes for ferritin is found in L. monocytogenes, which is a major cold shock protein and is overexpressed after heat shock or chemical stress (Dussurget et al., 2005). Apart from protection from stresses and iron binding activity Dussurget et al., (2005) reported that fri also plays a significant role in the production and/or stability of LLO, which in turn has an effect on L. monocytogenes virulence.

1.7 Host Susceptibility

Both humans and animals are susceptible to contracting infection from L. monocytogenes. Human listeriosis is overwhelmingly a foodborne disease and it has been estimated that 99% of all human listeriosis cases are caused by consumption of contaminated food products (Mead et al., 1999; Hain et al., 2006). The pathogen, if successful, will cross the intestinal barrier and disseminate to the liver and spleen from the mesenteric lymph nodes. From there it may reach the brain where it will cause meningitis or encephalitis, or the placenta which leads to abortions in pregnant women (Lecuit et al., 2007). In maternofetal or neonatal listeriosis occurring within the first week of life, it is
presumed that the fetus acquired the infection in utero through trans-placental migration of the organism from the bloodstream of the mother (Swaminathan and Gerner-Smidt, 2007). Sampling surveys by the CDC reported that 11% of the food samples collected during food monitoring programs were contaminated with *L. monocytogenes*, and that *L. monocytogenes* grew from at least one refrigerator sample of 64% of patients with active cases of listeriosis (Lecuit et al., 2007). The majority of cases of listeriosis occur amongst individuals who are immunocompromised, pregnant, elderly or young. Conditions such as HIV-AIDS, diabetes and alcoholism increase the risk of contracting listeriosis (Lecuit et al., 2007). Infection of these groups is directly correlated to the virulence of the strain involved. Mortality rates in invasive listeriosis have been calculated to be between 20% and 40% (Garcia et al., 2008). Pregnancy and neonatal disease accounts for 10%-20% of the cases. Incidence of infection increases with age, with men being more susceptible after age 40 and women after age 55 (McLauchlin et al., 2004). Gerner-Smidt et al., (2005) reported a higher mortality rate in patients infected with strains of serogroup 4; 26% of patients infected with *L. monocytogenes* serogroup 4 died compared with 16% of patients infected with serogroup 1/2, once again indicating that serogroup 4 strains may be more virulent.

In contrast Nightingale et al., (2005) state that a substantial number of *L. monocytogenes* strains found in food secrete a truncated InlA during infection. This mutation leads to a reduced ability to infect epithelial cells. Several of the strains tested were recovered from human cases, which does suggest the possibility that those cases
might be associated with specific host factors that increase susceptibility, such as extreme immunosuppression.

1.8 *Listeria monocytogenes* presence and distribution

All members of the *Listeria* genus are widely distributed in nature and have been found in soil, water, vegetation, sewage, fresh and frozen meat and animal feed (Kérouanton et al., 2010; Graves et al., 2010; Linke et al., 2014; McLaughlin et al., 2011; Vivant et al., 2013). In the dairy farm environment *L. monocytogenes* has been isolated from soil, water, silage, and bulk tank milk (Santorum et al., 2012; Sauders and Wiedmann, 2007; Nightingale et al., 2004; D’Amico et al., 2008; Pritchard et al., 1995; Wiedmann et al., 1996.). This ubiquitous nature and persistence of the bacterium in vastly different environments represents a great threat to public health. The principal root of infection in humans is through contaminated foods. Other possible routes of contamination for humans include direct contact with the environment, via contact with infected animals and though cross infection between newborn infants (McLauchlin et al., 2004). Although pasteurization of milk has helped in the control of pathogenic bacteria in dairy products, outbreaks still occur through post processing recontamination and consumption of raw milk products (Van Kessel et al., 2011). Contamination of milk in the farm setting can occur from fecal contamination of teats, udder infections (mostly in sheep and goats), and milking equipment (D’Amico et al., 2008; Hunt et al., 2012).

Contamination of the bulk tank milk can happen either as a result of exogenous contamination via the milking equipment through fecal contamination during milking, or less frequently by an intramammary route following asymptomatic infection or mastitis.
Most surveys of bulk milk tanks report a contamination rate between 1-16% (Santorum et al., 2012). This difference can be an indicator of many factors including differences in animal husbandry practices, location and differences in herd size (Santorum et al., 2012). Antognoli et al., (2009) screened milk samples collected from 871 farms with a minimum herd size of 30 cows in 21 states. The study reported that practices that increased the potential for *L. monocytogenes* contamination of bulk tank milk were automatic take offs and open herd management. These authors reported that samples from the northeastern and southeastern U.S. were 6 and 4 times more likely, respectively, to be contaminated with *L. monocytogenes* when compared to the western U.S., a result that alludes to the importance of geographical location. In addition, the study reported that farms with >500 milking cows were 5 times more likely to have bulk tank milk contamination. D’Amico et al., (2008) investigated the prevalence of four target pathogens, one of which was *L. monocytogenes*, in raw milk destined for farmstead cheese production and reported that the incidence of this pathogen in milk samples was 2.3% (3 positive samples out of 133). The data from that study suggested that recontamination of bulk milk probably occurs from a separate source rather than persistent contamination. The prevalence of *Listeria* in milk samples reported in this study was in agreement with the levels that were reported in Europe (1-5.3%). Other studies have reported contamination rates of bulk milk tanks to be 6.1% (Vilar et al., 2007) and 6.5% (Van Kessel et al., 2004).

Presence of *Listeria* spp. in vegetation from agricultural areas has been reported to range from 9.7 to 44%, while for non-agricultural areas it ranges from 21.3 to 23.1% (Sauders et al., 2012). In agricultural soils *Listeria* spp. prevalence can range from 8.7 to
51.4%, while in non-agricultural soils the species ranges from 15.2 to 43.2% prevalence (Sauders et al., 2012). Although the most prominent species found in food processing environments are *L. monocytogenes* and *L. innocua*, Sauders et al., (2012) tested 1805 samples from soil, water, and other environmental samples, that resulted in 442 positive *Listeria* isolates, from both urban and agricultural areas. These authors concluded that *L. innocua* and *L. monocytogenes* were associated with urban environments while *L. seeligeri* and *L. welshimeri* were associated with natural environments.

Lyautey et al., (2007) reported that there is a significant link between contamination of water sources and proximity to dairy farms. Farm practices such as untreated manure spreading for the purpose of fertilization of pasture fields can also lead to contamination of soils and silage (Santorum et al., 2012). Contaminated soil dust has also been found to contain *L. monocytogenes*, suggesting that the pathogen can also be transferred by air (Korthals et al., 2008). Water sources can also be contaminated through domesticated animals or wild life including birds. Listeriosis has been confirmed to be potentially present in a variety of domestic and wild animals (Hunt et al., 2012).

Several studies have reported the presence of *Listeria* spp. in silage (Wiedmann et al., 1996; Nightingale et al., 2004; Ryser et al., 1997; Arimi et al., 1997; Vilar et al., 2007). Silage is made primarily from grass crops, but can be made also from many other crops including corn. Silage undergoes rapid anaerobic lactic fermentation that converts sugars to acids and exhausts any oxygen present in the crop. Due to the acid production the pH in silage drops rapidly to less that 4.5 (Fenlon and Shepherd, 2000). If fermentation occurs properly, the conditions present inhibit the growth of spoilage bacteria and *Listeria* spp. If
aerobic conditions are still present, the fermentation does not occur properly and as a result the pH will not reach 4.5 or below, and conditions then become favorable for *Listeria* spp. growth (Ryser et al., 1997). Vilar et al., (2007) reported that 6% of the silage samples from 83 farms were found to be positive for *L. monocytogenes* and that low quality silage (indicated by pH > 4.5) was almost 5 times more likely to be found positive for the presence of *L. monocytogenes* when compared to high quality silage (indicated by pH <4.5). Field crops can be contaminated with *L. monocytogenes* through the practice of manure fertilization, or simply due to presence of *Listeria* in soil. Soil borne *Listeria* have been reported to be persistent in the environment for lengthy periods of time (Donnelly, 2001). Ferreira et al., (2011) was not able to report any unique genotypic or phenotypic characteristics for persistent *L. monocytogenes* strains and concluded that persistence in *L. monocytogenes* is diverse. Fermentation will also not occur properly if there is growth of molds and yeasts. The growth of the molds will result in raising the pH level of the silage to the range in which *Listeria* spp. can survive. Fenlon (1985) reported in his study that *L. monocytogenes* is more often found in silage stored in bales than in bunk or silos due to the increased surface area exposed to aerobic conditions.

Association between silage and listeriosis in ruminants was first recognized in 1922 in Iceland (Ryser et al., 1997). The presence of *L. monocytogenes* recovery from improperly fermented silage has been well documented (Ryser et al., 1997; Hunt et al., 2012; Arimi et al., 1997; Nightingale et al., 2004; Sanaa et al., 1993; Santorum et al., 2012). Poor quality silage is commonly contaminated with *L. monocytogenes* and following ingestion ruminants can transmit the pathogen, either as asymptomatic carriers or by
shedding it in their milk as a consequence of clinical or subclinical mastitis (Linton et al., 2008). Wiedmann et al., (1996) was able to establish the causal relationship between silage disease outbreaks by identifying the same ribotype of *L. monocytogenes* from clinical samples and corresponding feed samples. Recently a case study investigating an outbreak in cattle, caused by an unusual 4b lineage III *L. monocytogenes* strain, tested the pH of the barley silage that was used as a feed and found its pH to be 7.0, a clear indication of improper fermentation (Bundrant et al., 2011). Silage crops that are grown on contaminated land can initiate a new cycle of silage contamination and fecal shedding by ruminants that consume such silage may ensue (Santorum et al., 2012).

Presence of *Listeria* in silage, milk or in the farm environment can potentially increase the presence of *L. monocytogenes* in raw milk cheese making (West, 2008). However, Rudolph and Scherer (2001) reported that cheese made from pasteurized milk has a higher incidence of *L. monocytogenes* (8%) than cheese made from raw milk (4.8%). A study by Manfreda et al., (2005) tested 1656 Gorgonzola cheeses after packaging and at the end of shelf life and reported *L. monocytogenes* contamination rates of 2.1 and 4.8% respectively. In addition, Donnelly (2005) reported that when outbreaks of human illness that involve consumption of raw milk cheese are reviewed, presence of factors other than raw milk use contribute to the presence of the pathogen. Based upon this alternative view of the science of cheesemaking, the Specialist Cheesemakers Association (UK) has developed and published the specialist cheesemakers assured code of practice (SCA, 2013) that provides guidance to members on how to produce safe raw-milk products, and the American Cheese Society has advocated the use of Hazard Analysis Critical Control Points.
(HACCP) to insure product safety. These protocols outline good practice from pasture to packaging. Cheesemakers are advised, for example: to work with premium quality milk; to monitor milk quality through the measurement of microbial levels and somatic cell counts; to make cheese within the shortest possible time from milking (36 h maximum); to maintain clean cheesemaking and aging environments; practice good personal hygiene; monitor relevant times, temperatures, and acidity levels during production; to take sanitary measures of end products in order to detect emergent problems; and to maintain good batch records from production to point of sale (West, 2008).

Clinical cases of listeriosis in cattle are very rare, about 4 in 1000 (Erdogan et. al. 2001), which makes the detection of the pathogen even more difficult. In addition, the cattle might be healthy enough to withstand the attempt of the pathogen to infect them but the pathogen will be shed in their fecal matter in a process termed as transient shedding (Lyatey et al., 2007). Vilar et al., (2007) reported that 9.3% of fecal samples from milking cows from 97 farms tested positive for *L. monocytogenes*. Nightingale et al., (2004) conducted a study that investigated the ecology and transmission of *L. monocytogenes* in the farm setting through fecal, feed, soil and water samples. The study’s main conclusions were that presence of *L. monocytogenes* differs between small-ruminant and bovine farms, with bovine farms having 22.2% of samples testing positive for the pathogen as compared to 16.8% positive for small-ruminant farms. Among bovine farms, presence of *L. monocytogenes* in fecal matter was 27.8% and in soil samples it was 35.3%. *L. monocytogenes* prevalence was higher in case farms when compared with control farms (13.8% in fecal matter and 14.6% in soil samples). In addition, the study concluded that
cattle contribute to amplification and dispersal of *L. monocytogenes* into the farm environment and that ribotypes that are associated with human listeriosis cases, (DUP-1038B, DUP-1042B and DUP-1044A) were commonly present on ruminant farms.

Factors that can influence the survival of *Listeria* in soils include temperature, soil type, competing biota and farming practices (Vivant et al., 2013). Soils with low pH (<4) collected from forests showed a lower survival of the pathogen. This effect can be related to the fact that pH, like in cheeses, acts as a barrier to the pathogen growth and survival and limits its growth but it does not completely eradicate the pathogen from the soil (Jamieson et al., 2002). *L. monocytogenes* was reported to be negatively affected by higher, close to neutral pH in soil than acidic pH. This effect was reported to be related to the clay content of soils (Locatelli et al., 2013). Soil type has been shown to have an effect on the survival *L. monocytogenes*. Greater survival was observed in garden soil, sandy loam and clay loam soils when compared to sandy and clay soils (Vivant et al., 2013).

Long term survival of *L. monocytogenes* in acidic soils can be due to the fact that the acidic environment activates the glutamate decarboxylase system (GAD) which has been shown to be responsible for the survival of the microorganism under extreme acidic conditions (pH 2.5). The GAD system consists of a glutamate decarboxylase enzyme (gadD2) and a glutamate antiporter that act in concert to reduce acidification in the cytoplasm of the cell (Chaturongakul et al., 2008).

Temperature is another factor that influences the survival of the pathogen in soil. Higher temperatures (25 and 30°C) tend to not support the survival and growth of *L. monocytogenes*. However in a study by Welshimer conducted in the 1960’s, *Listeria* could
still be recovered after 67 days post inoculation. This result supports later studies that suggested that *Listeria* cannot be completely eradicated, that the survival in soil depends on previous exposure to adverse conditions, and that survival depends on the strain of the isolate (McLaughlin et al., 2011). McLaughlin et al., (2011) tested the survival of *L. monocytogenes* and *L. innocua* at three different temperatures, 25°C, 30°C, and 8°C using sealed and open soil tubes. The authors concluded that at 25°C and 30°C *L. monocytogenes* survived poorly in soils and were undetectable in soils past day 8. In contrast, the same *Listeria* strains were recovered at a level of 1.2 x 10³ cfu/g on day 14 from inoculated soil tubes that were incubated at 8°C. When the same strains were grown in BHI broth, 30°C was the optimum temperature where growth potential was the best. Although it was shown that there is a temperature effect, moisture content was not shown to have as strong an effect on the survival of the pathogen for the first 7 days.

Linke et al., (2014) investigated the presence of *Listeria* spp. from 10 different soil sites located at different altitudes. The authors hypothesized that higher elevation will promote higher survival of *L. monocytogenes* due to the ability of the pathogen to survive cold temperatures. *Listeria* spp. were found in 30% of 467 soil samples, with *L. monocytogenes* found in 6% of the soil samples. Interestingly, higher diversity of *L. monocytogenes* isolates and detection occurred after flooding events. Linke et al., (2014) also stated that *L. monocytogenes* presence and antibiotic resistance was higher in samples close to agricultural areas. Strawn et al., (2013) investigated the presence of *L. monocytogenes* in soils from produce fields and reported that 17% of the field samples tested positive for the pathogen. Interestingly, similar to the study by Linke et al., (2014),
Strawn et al., (2013) reported that detection of *L. monocytogenes* was increased after irrigation, wildlife presence or soil cultivation if those events occurred within 3 days of sample collection.

Lastly soil survival can also be associated with the ability of *L. monocytogenes* to associate with bacteriophagous organisms and nematodes. The pathogen can survive endocytosis by protozoa and replicate within the cytoplasm. After 8-14 days *L. monocytogenes* causes lysis of the host cell (Vivant et al., 2013).

1.9 Methods of detection and identification (speciation)

Identification of *L. monocytogenes* is extremely important both for prevention and control efforts. There are several ways to identify the pathogen in food and clinical samples, both through use of cultural based and molecular based methods (Jadhav et al 2012).

1.10 Culture based methods

Culture based detection methods rely on enrichment media and resistance of *Listeria* spp. to selective compounds that suppress the growth of background contaminants (Donnelly, 2002; Jeyaletchumi et al., 2010). Selective agents commonly used in enrichment/plating media include acriflavin, nalidixic acid, lithium chloride, moxalactam, phenylethanol and cycloheximide (Donnelly, 2002; Beumer et al., 2003). The most widely used protocols for detection of *Listeria* spp. are the ISO 11290 standard; the FDA-BAM method for isolation of *Listeria* spp. from dairy products, seafood and vegetables; and the USDA-FSIS methods for meat and poultry products as well as from environmental samples (Jeyaletchumi et al., 2010; Donnelly, 2002; Beumer et al., 2003; BAM; MLG; Gasanov et
al., 2005, Hitchins and Jinneman, 2011). These methods require use of a double enrichment procedure, with the primary or pre-enrichment broth containing lower amounts of selective agents to aid in the resuscitation of potentially injured cells (Beumer et al., 2003). The three selective agents used in these broths are acriflavine, nalidixic acid, and cycloheximide (Beumer et al., 2003, Donnelly, 2002). Inhibition of RNA synthesis and mitochondriogenesis is affected by the presence of acriflavin. The concentration of acriflavin in the media ranges from 10 to 25 mg/l (Beumer et al., 2003). The use of acriflavin has both direct and indirect effects on the isolation of *L. monocytogenes*; in contrast, there is no effect observed on *L. innocua*. Increasing acriflavin concentrations affects both lag time and generation time of *L. monocytogenes*. At low pH values (pH<5.8), acriflavin binds protein, which results in decrease of activity which in turn results in better growth of *L. monocytogenes* (Beumer et al., 2003). Nalidixic acid inhibits DNA synthesis of cells through inhibition of a DNA gyrase subunit and topoisomerase IV and is added to enrichment media to inhibit growth of Gram positive microorganisms. Nalidixic acid is usually combined with cycloheximide, which inhibits protein synthesis in eukaryotic cells by binding to the 80s ribosomal RNA. Higher concentrations of cycloheximide in selective media inhibit the growth of yeasts and molds, while neither nalidixic acid nor cycloheximide have an effect on the growth of *Listeria* spp. (Beumer et al., 2003). To counter this effect, the FDA method does not add the antibiotics in the first enrichment broth to allow injured cells to recover, while the ISO 11290 method uses half Fraser broth which contains half the concentration of the antibiotics (Donnelly 2002; Gasanov et al., 2005).
The primary enrichment broth used in the FDA method employs *Listeria* Enrichment broth (LEB) and an incubation period of 48h at 30°C. After incubation, enriched samples are plated onto differential media such as Oxford, PALCAM, LPM agar, MOX agar, and chromogenic media including ALOA, Rapid L’mono and CHROMagar™ (Jadhav et al., 2012; Gasanov et al., 2005). Oxford and PALCAM agar plates are incubated at 35°C for 24-48h, with the optional use of a CO₂ atmosphere (Donnelly, 2002). Lithium chloride phenylethanol moxalactam (LPM) is another media used for the identification of *Listeria* spp. LPM plates were viewed with Henry illumination, but use of chromogenic media has replaced this technique (Donnelly, 2002, Beumer et al., 2003). Under this procedure *Listeria* colonies look gray to light blue. Addition of esculin and ferric iron to LPM media eliminates the need for Henry illumination (Donnelly, 2002). In Oxford and PALCAM plates *Listeria* colonies develop a black halo (Donnelly 2002). ISO 11290 method utilizes half strength Fraser broth for the initial 24h of enrichment and full strength Fraser broth for further enrichment. The broth contains esculin that causes blackening of the broth in the presence of *Listeria* species due to the activity of β-D-glucosidase. Both broths are then plated on PALCAM or Oxford media (Gasanov et al., 2005). Although identification of *Listeria* spp. was possible, the methods lack the ability to distinguish between pathogenic and non-pathogenic strains. Use of blood-containing media allowed the separation between hemolytic species (*L. monocytogenes*, *L. ivanovii*, and *L. seeligeri*) and non-pathogenic species (*L. innocua*, *L. grayi* and *L. welshimeri*) (Beumer et al., 2003). Other methods used to distinguish between *Listeria* spp. are the CAMP reaction (Beumer et al., 2003) and xylose fermentation (Beumer et al., 2003; McLauchlin 1997).
The USDA method also uses a double enrichment process with a modified University of Vermont Medium (UVM) as the primary broth that contains actiflavin and naladixic acid. The secondary enrichment broth is Fraser broth, and then samples are plated on MOX agar supplemented with moxalactam and colistin sulphate. The AOAC/IDF method 993.12 is the preferred method for dairy products. The method has a specific instruction for sample preparation and uses a primary enrichment broth supplemented with acriflavin and nalidixic acid incubated for 48h at 30˚C. Samples are then plated onto Oxford agar (Gasanov et al., 2005).

More recently, detection of *Listeria* spp. on agar plates employs the use of chromogenic media. *Listeria* Ottavani and Agosti (ALOA) (Jadhav et al., 2012; Beumer et al., 2003) CHROMagar™ (Beumer et al., 2003) and RAPID’Lmono (Jadhav et al., 2012) are chromogenic media. ALOA is dependent on the β-glucosidase activity of *Listeria* spp. which cleaves the chromogenic substrate producing blue/green colonies. Lecithin present in the media is hydrolyzed by phosphatidylinositol phospholipase C (PIPL-C), synthesized only by *L. monocytogenes* and *L. ivanovii*, which create an opaque halo around the hemolytic species (Jadhav et al., 2012, Jeyaletchumi et al., 2010).

Cultural methods are extremely laborious as they require the random selection of a number of colonies from a single sample, and depending on the method used; it could take up to 10 days to confirm a positive sample (Jany and Barbier, 2008; Jadhav et al., 2012). Other disadvantages include false negative results, especially when using Fraser broth as the secondary enrichment broth, which is used in the USDA procedure (Donnelly, 2002). In addition phenotypic changes due to environmental selection, contaminating bacteria and
atypical reactions by atypical strains can also provide false negative results (Jadhav et al., 2012).

1.11 Immuno-based techniques

Immuno-based methods used for the detection of *Listeria* spp. require the use of antibodies that can be used to detect either whole cells or specific cellular components (Jadhav et al., 2012). Enzyme linked immunosorbent assay (ELISA) (Jadhav et al., 2012, Beumer et al., 2003) and immunomagnetic separation (IMS) (Jadhav et al., 2012) are two immuno-based techniques that can be used for the detection of *L. monocytogenes* in samples. ELISA uses anti-*Listeria* antibodies, which are immobilized to a microtiter well, along with secondary antibodies linked to an enzyme. An assay based on the ELISA method developed for *L. monocytogenes* by Magliulo et al., (2007), uses a primary monoclonal antibody that is recognized by the pathogen. Subsequently, a peroxidase-labeled polyclonal antibody against the target organism is added and the peroxidase activity of the bound polyclonal labeled antibodies was measured with the use of an enhanced luminol-based chemiluminescent cocktail using a low-light charge-coupled imaging apparatus. The method has a detection limit between $10^4$ to $10^5$ cfu/ml. These methods are able to produce results in 30-50h, are easy to interpret, however they are less sensitive when compared to cultural methods (Jadhav et Al., 2012; Ryser and Marth, 2007). A commercial kit that was created based on the ELISA principals, VIDAS® LMO2 (BioMérieux), was used by Ueda and Kuwabara, (2010) to detect *L. monocytogenes* 4b strain in foods and was observed to be faster than other methods, producing results in 70 min. However the study showed that it was not as sensitive as the method used by Magliulo
et al., (2007) and was able to detect $10^6$ cfu/ml in acidic foods like yogurts and fruit juices. In general, cultural methods have a sensitivity of detection of $10^4$ cells/ml, while ELISA sensitivity ranges between $10^5$-$10^6$ cells/ml. The IMS method uses paramagnetic polystyrene beads that possess pathogen specific antibodies covalently linked to the bead surface. *Listeria* antigens bind to the antibody in the presence of a magnetic field (Wadud et al., 2010). IMS methods when in combination with PCR detection has been shown to have a detection limit of 1 cfu/g post-enrichment (Jadhav et al., 2012).

### 1.12 Molecular methods of detection

Use of molecular methods for detection of pathogens is favored over cultural and immuno-based methods (Deng et al., 2010) due to the long culture periods which are time consuming (Jany and Barbier, 2008), labor intensive or involve repetitive multiple steps, which can introduce errors (Jadhav et al., 2012; Beumer et al., 2003) and the possibility of false positive results (Donnelly, 2002).

### 1.13 Nucleic acid probes

Nucleic acid probes can be segments of DNA or RNA labeled with radioisotopes, enzymes or chemiluscent reporter molecules that bind to complementary nucleic acids with high specificity. Although these methods are more specific that serological or biochemical methods, their effectiveness is limited by the fact that they do not involve nucleic acid amplification (Jeyaletchumi et al., 2010). Commercial formats include GENE-TRAK® *Listeria monocytogenes* Assay (Gene-Trak systems, Framingham, MA, USA) (Allerberger, 2003), GeneQuench® *Listeria monocytogenes* Test Kits (Neogen, Lansing,
Michigan, USA) (Janzten et al., 2001) AccuProbe® (Gen-Probe, San Diego, CA, USA) (Allerberger, 2003).

**1.14 Polymerase Chain Reaction Based Methods (Conventional PCR, RT-PCR, Multiplex PCR, Real Time-PCR)**

Conventional polymerase chain reaction (PCR) targets specific genes such as *hly*, *inlA, inlB, iap*, intergenic regions, 16S and 23S rRNA genes, invasion associated protein p60, aminopeptidase C, phospholipase C protein, fibronectin binding protein, and dth-18 (delayed type hypersensitivity) protein (Jadhav et al., 2012; Brehm-Stecher and Johnson, 2007; Jeyaletchumi et al., 2010; Rodríguez-Lázaro et al., 2004). PCR techniques are used more frequently than cultural procedures due to the fact that they are simple and can provide quick results (Jeyaletchumi et al., 2010). However PCR cannot distinguish between live or dead cells or viable but not culturable cells (VBNC) (Aznar and Alarcon, 2003), metabolically injured, stressed cells, or reliably detect low levels of *L. monocytogenes* (Jadhav et al., 2012). In addition, PCR can be affected by the presence of inhibitors such as phenolic molecules, nuclease or selective agents used in enrichment procedures, DNA yield, chaotropic and chelating agents, and genetic variation of the targeted sequence (Jany and Barbier, 2008; Jadhav et al., 2012). Usually an internal amplification control (IAC) is added to the reaction to act as an indicator for PCR inhibitory agents (Cocolin et al., 2011). Compared to cultural methods, DNA amplification can produce an overestimation of health risk associated with food due the high stability of DNA molecules that can persist in the sample even after cell death (Cocolin et al., 2011; Jadhav et al., 2012). A positive sample through DNA amplification does not necessarily mean that
the organism is alive and in required concentrations that makes that organism a public health risk (Cocolin et al., 2011).

Reverence transcriptase PCR (RT-PCR) is able to differentiate between live and dead cells (Jadhav et al., 2012). mRNA has a shorter half-life compared to DNA and degrades faster after cell death. Although RT-PCR enables differentiation between live and dead cells it has several limitations that make it both expensive and unreliable. mRNA extraction is a tedious process that can be interfered with by the presence of DNA. Additionally, mRNA is sometimes only produced when the cell is exposed to certain environmental stimuli such as temperature changes (Brehm-Stecher and Johnson, 2007). To bypass this limitation Pan and Breidt, (2007) investigated the use of propidium monoazide (PMA) and ethidium monoazide (EMA) in cell aliquots of both live and dead cells at different time intervals of up to 2h. The bacterial cells were treated with either PMA or EMA prior to DNA extraction. These dyes have the ability to enter dead cells but not live ones and can bind to double stranded DNA or RNA and once exposed to light they can form a permanent covalent linkage with the nucleic acids that can prevent PCR amplification. These authors found that PMA was not toxic to live cells and had a detection limit of $10^3$ CFU/ml while EMA was toxic to live cells.

Multiplex PCR is another PCR based method which allows the detection of multiple strains from the same species or multiple pathogens in a sample simultaneously (Ryu et al., 2013). The detection specificity of this method depends on the specific binding of the primer pair to the target sequence of the microorganism. Ryu et al., (2013) developed a multiplex PCR method that can distinguish between 5 different *Listeria* spp. including *L.*
monocytogenes by targeting different genes for each species. Multiplex PCR can detect between 1-100 CFU/ml Listeria (Jadhav et al., 2012), however similar to conventional PCR it can overestimate the presence of the pathogen because it cannot distinguish between live and dead cells.

Real time-PCR differs from other PCR techniques because the amplicon is observed as it accumulates. The procedure monitors the accumulation of fluorescence levels which in turn depend on the amount of the accumulated PCR product. The fluorescent molecule can be either a target specific probe labelled with a fluorescent dye together with a quencher molecule or can be a non-specific DNA binding dye. The method is highly sensitive, has the ability to detect trace amounts of target DNA, can be automated, and has the ability to quantify bacterial load without any post-PCR handling. However its disadvantages are that primer dimers can show fluorescence, it is highly dependent on primer concentration and design, and requires stringent quality controls.

1.15 DNA Microarrays

Microarrays are based on DNA or RNA hybridization (Volokhov et al., 2002). They can be used as an epidemiological tool, to investigate microbial evolution, and can serve as a diagnostic tool. There are 2 types of microarrays: PCR-based and oligonucleotide based (Gasanov et al., 2005). Reliance of the method on PCR amplification may introduce bias into samples and cross hybridization may also occur between similar sequences which complicates the analysis of variants and single nucleotide polymorphisms (SNP’s) (Hurd and Nelson, 2009). Volokhov et al., (2002) developed a PCR-Microarray method that was able to differentiate between Listeria sp. Microarrays are highly specific assays that utilize
a species specific probe to a complimentary single stranded DNA on the target organism however the method requires a prior knowledge of the genome (Hurd and Nelson, 2009). Advantages of oligonucleotide based microarrays over PCR based microarrays include the reduced incidence of cross contamination and cross hybridization, the use of synthetic oligonucleotides instead of RT-PCR amplification and products purification, and the ability to perform hybridization directly by using total bacterial RNA previously labeled (Gasnov et al., 2005). Call et al., (2003) and Doumith et al., (2004) have used microarrays to subtype and investigate phylogenetic relationships among L. monocytogenes strains belonging to the serotypes 1/2a, 1/2b, 1/2c, and 4b.

1.16 Subtyping

Current subtyping methods such as pulse field gel electrophoresis (PFGE) have been valuable tools for the surveillance and detection of foodborne pathogens, however there are several areas where these subtyping methods are not able to provide us with high discriminatory power. For example, these methods do not have high discriminatory power for highly clonal pathogens. Whole genome sequencing can overcome this issue. In addition WGS can assist with grouping pathogenic isolates into epidemiological relevant groups, which will help with outbreak investigations (Bergholz et al., 2014). Most of the current subtyping methods such as PFGE and MLST capture only a small proportion of the true genetic content, so it is still difficult to interpret robust data sets such as whole genomes when all previous characterizations have been comparatively limited in detail (Gilmour et al., 2010).
Pulsed-field gel electrophoresis (PFGE) has been used for the molecular subtyping of *L. monocytogenes* as part of the PulseNet system since 1998. However, PFGE patterns are complex and not easy to interpret, the method is labor-intensive and time consuming, cannot be adapted to target specific polymorphisms of interest, and can be affected by relatively unstable genetic elements, such as plasmids and phages (Ducey et al., 2007). In addition there are possible inter laboratory variations due to changes in experimental conditions (Jadhav et al., 2012).

A number of multilocus sequence typing (MLST) methods have been described for *L. monocytogenes* (Knabel et al., 2012; Nightingale et al., 2006; den Bakker et al., 2014). MLST depends on multiple gene fragments or genes to differentiate between subtypes (Jeyaletchumi et al., 2010). The method relies on amplification of seven loci from housekeeping genes that are analyzed for nucleotide differences (Jadhav et al., 2012). Although this method has been shown to be comparable to amplified fragment length polymorphism (AFLP) (Parisi et al., 2010), but was shown to be more discriminatory when compared against the PFGE method. However those differences could be because of the different genes that are targeted from those methods (Jadhav et al., 2012). MLST is considered an expensive and time-consuming method because it requires numerous sequencing reactions per isolate and cannot be multiplexed and it does not have enough discriminatory power for 4b *L. monocytogenes* serotypes (Jadhav et al., 2012), which are among the serotypes often implicated in outbreaks. Direct interrogation of single-nucleotide polymorphisms (SNPs) could offer a more efficient alternative for DNA
sequence-based subtyping based on the fact that the majority of sites sequenced for MLST are invariant (Ducey et al., 2007).

Ribotyping is another method that is used extensively for molecular subtyping. The method was originally used to investigate phylogenetic relationships and is based on variations on ribosomal genes or proteins (Gasanov et al., 2005). Several studies have used this method to identify *L. monocytogenes* strains involved in outbreaks (Bundrant et al., 2011) and to identify single source clusters among human listeriosis cases (Sauders et al., 2003). Initial genomic DNA digestion from restriction enzymes such as *Eco*RI, *Pvu*ll or *Xho*I generate as many as 500 small fragments. Southern blot is then utilized to detect digests that can hybridize with specific genes targeting the genes that code for ribosomal RNA (Jadhav et al., 2012). This method was commercialized by the DuPont Corporation with as the Riboprinter Microbial Characterization System ® (Qualicon Inc., Wilmington, DE) (Sauders et al., 2003). Previously, there was interlaboratory differentiation due to gel to gel variations (de Cesare et al., 2007) but automation of this procedure has eliminated, to some extent, these issues. However, the cost of running multiple samples has increased and ability to compare isolates is limited to current libraries that are continually updated. Although this method is highly reproducible and discriminatory, studies have reported that the power of differentiation among *Listeria* isolates is lower than other subtyping methods (Jadhav et al., 2012; Gasanov et al., 2005; Graves et al., 2007).

Multilocus variable number tandem repeat (MLVA) is another method of typing that detects variations in the number of tandem repeats from a particular locus in the genomic DNA of the microorganism (Murphy et al., 2007). This method is rapid, takes
about one day to be completed, and is simple, reliable and economical. A major drawback for this method is that the primers require standardization and optimization. Chen et al., (2011) used this method to subtype *L. monocytogenes* isolates from spiked samples and reported that this method has a detection power of 1-5 cfu/25g of food. Miya et al., (2008) compared MLVA, MLST and PFGE ribotyping techniques against 4b isolates and reported that MLVA had the highest discriminatory power followed by PFGE and MLST.

Lately researchers have an additional tool in understanding the genome of this pathogen and that is the next generation sequencing (NGS) platforms that are able to manage large data sets of parallel short read DNA sequences (Hurd and Nelson, 2009). NGS platforms currently most commonly used include Roche 454 (GS-FLX, GS-FLX+), Illumina (GA, GAII, HiSEQ, MISEQ) and Applied Biosystems (ABI SOLiD) (O’Sullivan et al., 2013). Recently bench top versions of these platforms are powerful enough for single organism sequencing, producing from 2Gb (Ion Torrent) to 35 Mb (454 GS Junior) (Gilchrist et al., 2015). One of the NGS technologies includes pyrosequencing. This was the first sequencing platform to be commercially available and uses emulsion PCR library fragments affixed to microbeads. Pyrosequencing techniques employ coupled enzymatic reactions to detect inorganic pyrophosphate (PPI) that are released as a result of nucleotide incorporation by DNA polymerase. Released PPI is converted to ATP by ATP sulfurylase, which provides the energy for luciferase to oxidize luciferin and generate light. Unincorporated nucleotides are degraded by apyrase before the addition of the next nucleotide (Ronaghi and Elahi, 2002). The Illumina genome analyzer (Mellmann et al., 2011) is yet another useful platform that was commercially released in 2006 (O’Sullivan
et al., 2013). This method is similar to the pyrosequencing platform but instead of beads, amplification occurs by single stranded DNA fragments that are attached to a flow cell via an adaptor molecule. Similar to the 454 pyrosequencing method, this system also allows sequencing by synthesis approach but instead of the nucleotides being added individually, all four are added at the same time together with a DNA polymerase (O’Sullivan et al., 2013). A third platform is called SOLiD and it is offered by Applied Biosystems and was commercially released in 2007. This method employs the bead technology like the 454 pyrosequencing, with the main difference being that it employs sequencing by ligation rather than synthesis. Due to each base being interrogated twice in sequential rounds of ligation it increases the accuracy level by its ability to minimize base calling errors and it is favored for genome resequencing or polymorphism analysis (Hurd and Nelson, 2009; O’Sullivan et al., 2013). Unnerstad et al., (2001) utilized pyrosequencing successfully to group L. monocytogenes isolates into different categories based on nucleotide variations of the inlB gene, showing the potential of this method to be used for subtyping of L. monocytogenes strains.

1.17 Comparison of cultural vs. culture independent methods

Traditionally, determining the composition and frequency of the microbial population in any matrix has involved the use of culture based methods. This included a large list of selective media and conditions with the hope that the majority of the organisms that were present in the sample will be accurately represented. However, cultural methods are time consuming, labor intensive and can only be used for the culturable microbiota that is present in sufficient numbers to out compete any other microorganism (Golsteyn et al.,
When applied to highly microbial diverse food product such as a natural cheese, cultural methods will favor the growth of microorganisms such as *Lactobacillus, Streptococcus, Enterococcus, Lactococcus, Leuconostoc, Weisella and Pediococcus*. However strains like *Propionibacterium, Staphylococcus, Corynebacterium, Brevibacterium* and in general other less abundant strains are lost or their detection becomes more difficult and they are not accurately represented (Quigley et al. 2011).

For the above reasons, culture independent methods are employed when assessing the microbial community of a food product such as a cheese rind. These methods are mainly DNA based and enable the simultaneous characterization of whole ecosystems and the identification of many species. These methods are more sensitive than the culture dependent methods, less labor intensive and more rapid. However, there are several limitations associated with these methods. The first one is that some methods cannot differentiate between live and dead cells (eg. PCR methods). These are usually DNA based methods. RNA based methods can, on occasion, facilitate the identification of the microbes in the sample and can be used as a target for live cells (Quigley et al., 2011).

A second limitation of culture independent methods is the quality of the DNA extraction. This is especially true when the sample being extracted represents the total microbial population, as is the case in high throughput sequencing experiments. DNA extraction can be improved by the introduction of chemical or mechanical lysis, protein digestion and DNA precipitation.
Lastly another limitation is the target sequence selected for amplification. For bacterial populations, traditionally the V3 or V4 region of the 16s RNA is most commonly amplified, although there are suggestions that other regions can also be used. Apart from the 16s RNA region, for *Listeria* detection, one can also target specific genes like *sig* B (Moorhead et al. 2003), *pheS* (Quigley et al., 2011), *iap* (Lazaro-Rodriguez et al., 2004), and listeriolysin O (Thomas et al., 1991).

An advantage of the WGS approach is the ability to construct the evolutionary model of an outbreak. Within approximately three days of isolation of the strain, draft genome sequences can be available that are suitable to begin comparative analyses such as genome alignments. Whole genome sequencing has therefore enabled robust real-time characterization of virulence determinants and genetic diversity within a natural *Listeria* spp. population (Gilmour et al., 2010).

**1.18 Use of Whole Genome Sequencing in Epidemiological Investigations**

Use of Next generation sequencing was first used to identify an outbreak in 2011, previously involving only one case of HUS associated with *E. coli* O104:H4, which took place in 2001 in Germany (Mellmann et al., 2011). Although the initial typing for this outbreak was done using traditional MLST typing method, whole genome sequence (WGS) exposed major differences in both chromosomal and plasmid content between the 2011 isolate from the 2001 isolate and this finding was also supported by Hao et al., 2012.

Traditional methods such as PFGE and MLVA have been extremely valuable for surveillance and detection of foodborne outbreaks but they show limited discriminatory power for highly clonal pathogens (Bergholz et al., 2014; O’Flaherty et al., 2011). WGS
has the power to overcome this hurdle and group isolates into epidemiologically relevant
groups. In addition to epidemiological data, WGS can provide rapid generation of whole
genome sequence data that can help identify targets that could be used to develop assays.
This ability is especially important for specific outbreak strains, or like in the case of the
*E. coli* O104:H4 case described earlier, develop new tests for detection (Sabat et al., 2013;
Bergholz et al., 2014). However the limitation of this procedure is that in order for
identification of virulence factors to occur, an extensive knowledge of the genetic basis of
the virulence needs to exist *a priori* (Gilchrist et al., 2015). As the price of a single
microorganism genome sequencing continually decreases ($100-150 USD) there is
increased interest in incorporating this technology for food pathogen detection as well as
epidemiological studies (Sabat et al., 2013).

Although the use of NGS provides valuable information that previously was not
available in such a short time period, there are still major barriers that need to be addressed
before these techniques can be incorporated in foodborne pathogen detection. The inability
to distinguish between a live cell, a dead cell or an injured cell has been described
previously, and like in other targeted gene methods, it poses the possibility of false
positives that can have detrimental effects on the industry and the consumer (Jadhav et al.,
2012; Bergholz et al., 2014). The need for computer platforms that are operator friendly,
powerful enough to handle the massive data bases that are created, and are easily
interpreted, still exists. In addition there is no consensus on how these data will be stored
or used by regulatory authorities like FDA and CDC during inspections or outbreak
investigations. It is almost certain that within the volume of data collected there will be
some sequence data that might be misconstrued as indicating a health hazard (Bergholz et al., 2014).

Further limitations of the WGS approach are the need for highly trained bioinformatics professionals that smaller companies will not be able to afford, lack of standardized and validated protocols like the ones already existing for PFGE, lack of reference databases, and lastly a large investment of resources that also have their own limitations (Sabat et al., 2013; Gilchrist et al., 2015; Wiedmann et al., 2011). In addition food borne pathogens are usually found at very low numbers, which poses its own limitations for epidemiological studies (Gilchrist et al., 2015).

1.19 Notable Listeriosis Outbreaks

Several outbreaks have shown the importance of controlling *L. monocytogenes* in foods. One of the first documented outbreaks occurred in 1981 in Canada involving 41 listeriosis cases (34 perinatal and 7 adult) (Schlech et al., 1983; Conly and Johnston, 2008). This outbreak was the first documented outbreak for this organism linked to foods and it involved the consumption of coleslaw. Cabbage that was used for making the coleslaw originated from a farm that had sheep that were infected with the pathogen. The sheep manure was used to fertilize the fields which resulted in the contamination of the cabbage.

A second outbreak that resulted in 49 cases of listeriosis occurred in Massachusetts in 1983. Of the cases reported, 29% were fatal (Fleming et al., 1985). The cause of the outbreak was identified as consumption of improperly pasteurized milk from a single cooperative of 450 farms in Vermont. In the investigation that followed, there were several
serotypes of *L. monocytogenes* that were isolated from samples obtained from milk filters and milk samples prior to pasteurization. The serotypes implicated included serotypes 1a, 3b, 4a-b, and 4b (Fleming et al, 1985). One of those serotypes was identified through ribotyping as DUP-1042B which is still found in environmental samples from Vermont (Jeffers et al., 2001; D’Amico and Donnelly, 2008)

Following this outbreak, in 1985 in California there was a larger listeriosis epidemic that involved 142 cases with 88 deaths. The vehicle for this outbreak was a fresh Mexican style cheese, queso fresco. There were 5 *L. monocytogenes* serotypes identified among the 105 clinical isolates, 82% of which were serotype 4b (Linnan et al., 1988). For this outbreak FDA recalled approximately 500,000 lb. of products distributed in 26 states, the US protectorates of Guam, American Samoa, and the Marshall Islands (Ryser and Martin, 2007). The ribotype pattern associated with clinical cases in this outbreak was DUP-1038B (Jeffers et al., 2001)

Between the years 1983 and 1987 there was an outbreak of *L. monocytogenes* infection in western Switzerland which was associated with the consumption of a soft cheese, Vacherin Mont D’Or, produced from pasteurized milk (Bille et al., 1990; Büla et. al., 1995). There were a total of 122 cases of listeriosis that occurred, with 53% occurring in pregnant women and 47% occurring in non-pregnant adults. The overall case fatality rate was 28%, with 93% of the serotypes characterized as belonging to serotype 4b (Ryser and Martin, 2007). Further investigation of this outbreak resulted in isolation and characterization of the outbreak strain as a *L. monocytogenes* DUP-1038B, which is the same strain that caused the 1981 outbreak in Canada, discussed earlier. This ribotypes was
isolated from cheese surfaces and clinical cases, along with wooden shelves and brushes used in the ripening room. The levels found were of the order 10^4-10^6 CFU/g (Bille, 1990).

In 1995, L. monocytogenes was associated with the soft raw milk cheese Brie de Meaux, which became the first outbreak in France to be associated with a raw milk cheese (Ryser and Martin 2007). In 1994 there was an outbreak linked to chocolate milk in Illinois, US. The outbreak affected people attending a picnic at a Holstein cow show (Dalton et al., 1997). L. monocytogenes infected 45 healthy adults who drank chocolate milk that was heavily contaminated following pasteurization. Symptoms were those of a self-limited, febrile, diarrheal syndrome; there were no deaths (Wing and Gregory, 2002). The milk was cultured and found to be contaminated with L. monocytogenes, serotype 1/2b. The same strain of Listeria was isolated from the stools of some of the ill persons and from a tank drain at the dairy. The milk had been pasteurized after the chocolate flavoring had been added. After pasteurization it was pumped into a holding tank, which later on was reported to have a breach in its lining. As the tank was drained, this milk could re-enter the tank through the breach and contaminate the milk. The milk was pumped into sealed containers and placed in a refrigerated room. Milk cartons were transported unrefrigerated to the picnic where the milk was also served unrefrigerated. Two unopened cartons of chocolate milk, one from the picnic and one produced on the same day at the dairy, yielded 1.2×10^9 and 8.8×10^8 CFU of L. monocytogenes/ml, respectively (Dalton et al., 1997).

Between 2011 and 2015 there were 8 outbreaks reported by the CDC (2015). In 2011 a multistate listeriosis outbreak linked to cantaloupe involved a total of 147 persons infected with any of the five outbreak-associated subtypes of L. monocytogenes reported
to CDC from 28 states. Ages of the affected patients ranged from <1 to 96 years, with a median age of 78 years. Most ill persons were over 60 years old with 58% of ill persons being female. The outbreak caused 30 deaths and one miscarriage (Fang et al., 2013).

A non-dairy outbreak involved commercially packaged caramel apples and was spread among 12 states from October 2014 to January 2015 (CDC, 2014b). The outbreak involved a total of 35 cases with seven deaths and one fetal loss, with the ages of the ill persons ranging from 7 to 92 years and with 33% of the cases being female. Apart from the importance of the outbreak, this was also one of the first outbreaks that was investigated using both PFGE and WGS. The second outbreak involved bean sprouts that were produced by Wholesome Soy Products Inc. and the outbreak led to the shutdown of the company on October 2014. The outbreak only lasted for a few months, from August 2014 to September 2014, due to the short shelf life of the product (5 days). There were 5 cases reported and in this outbreak both PFGE and WGS methods were used to identify the strains that were collected from environmental samples. FDA also issued a report citing the company with 12 inspectional observations, citing the firm for numerous unsanitary conditions and poor equipment maintenance (CDC, 2014a).

In 2012, 22 people in 13 states were infected with *L. monocytogenes* from ricotta salata cheese that was imported from Italy (CDC, 2012). There were 4 deaths reported with the ill person ages ranging from 30 to 87 years, with 54% of the cases being female. For this outbreak PFGE methods were used to identify the *Listeria* strains. In 2013 Crave Brothers Farmstead recalled Les Frères, Petit Frère, and Petit Frère with Truffles cheeses, which are washed-rind Reblochon-style cow milk cheeses, due to possible contamination
with *L. monocytogenes*. From May 2013 to June 2013 there were 6 cases reported from 5 states with 83% of the cases being female and with ages ranging from 30 to 67 years old. There was one death and one miscarriage reported related to this outbreak (CDC-2012).

In 2013, there were two more outbreaks that involved Mexican style cheeses. The first outbreak involved 8 cases reported in two states with onset from August 2013 to November 2013, with no new cases reported beyond March 2014. Seven of the cases reported were hospitalized, all of which were of Hispanic ethnicity. There was one death reported that was associated with this outbreak and a recall was issued. The *Listeria* strains were identified using the WGS method. The second outbreak also involved commercially made Mexican style cheeses and fresh curds, which infected 5 people in 4 states. FDA performed PFGE and WGS to identify and track these *Listeria* isolates and a recall was issued. There was one death and 4 hospitalizations and all of the infected people were of Hispanic ethnicity (FDA, 2014c)

More recently an outbreak that was reported by the FDA and CDC was the Blue Bell ice cream outbreak. It involved 10 cases from 4 states with 3 deaths reported from the period February 2015 to May 2015. The facilities for this company are currently closed and similar to the outbreaks from 2014, FDA used both PFGE and WGS methods to identify and track the strains of *L. monocytogenes*. The use of WGS, according to the CDC, gives a more detailed DNA fingerprint than PFGE (FDA 2014c). This was the first outbreak where WGS was used to retrospectively associate *L. monocytogenes* isolates with clinical samples.
1.20 Predominant serotypes in animal vs human listeriosis

Differences in virulence between strains may influence infection and clinical outcome. Serotypes 1/2a, 1/2b, and 1/2c are the types most frequently isolated from food or the food production environment. However, more than 95% of infections in humans are caused by the three serotypes 1/2a, 1/2b, and 4b (Bundrant et al., 2011; Nightingale et al., 2005; Swaminathan and Gerner-Smidt, 2007). All human epidemic isolates cluster into lineage I, and none of the 21 human isolates examined by Wiedmann et al., (1997) fell into lineage III. Isolates from human sporadic cases were distributed between lineage I and lineage II (65 and 35%, respectively), while animal isolates appeared to encompass a more diverse population which spanned all three lineages (Jeffers et al., 2001). Knabel et al. (2012) reported that from 1988 to 2010 most outbreaks in Canada were predominantly caused by 1/2a serotype \textit{L. monocytogenes} strains. A majority of listeriosis outbreaks are caused by strains of serotype 4b. The rate of isolation of serotype 4b is higher among patients suffering from meningoencephalitis than in patients suffering from blood stream infection, indicating that strains of serotype 4b may be more virulent than saprophytic \textit{Listeria} associated with soils and green plant material (Swaminathan and Gerner-Smidt, 2007). Jeffers et al. (2001) reported in his study that the DUP-1042 strain, a 4b serotype \textit{L. monocytogenes}, was found in 5 of the \textit{Listeria} outbreaks between the years 1976-1990 including the 1983 pasteurized milk outbreak in Massachusetts, USA. The next most prominent ribotype that cause outbreaks during the same time period was DUP-1038 which is also a 4b serotype \textit{L. monocytogenes} strain.
Our understanding of the transmission of *Listeria* in ruminants is still limited (Bundrant et al., 2011). Bundrant et al., (2011) reported an unusual *L. monocytogenes* strain, (4b serotype, lineage III), that was implicated in an animal outbreak. During an outbreak, disease prevalence in cattle is usually low, between 8-10%, but it can reach up to 15%. Mortality rates in cattle range from 20% to 100%. Typical symptoms of encephalitic listeriosis include facial paralysis, drooling, circling, and recumbency (Bundrant et al., 2011).

1.21 Association of *Listeria* with cheese

According to the Quantitative Assessment of Relative Risk to Public Health from Foodborne *Listeria monocytogenes* Among Selected Categories of Ready-to-Eat Foods (FDA, 2003) cheese products can be divided into 6 different categories. These are i) fresh soft cheese (e.g. Queso fresco), ii) soft unripened cheeses, characterized as cheeses that have >50% moisture (e.g. cottage cheeses, cream cheese), iii) soft ripened cheeses, characterized as cheeses with >50% moisture but with an aging period of at least 60 days (e.g. Brie, Camembert, Mozzarella), iv) semi-soft cheeses, characterized with 39-50% moisture content (e.g. Monterey, Muenster), v) hard cheeses, characterized as cheeses with a moisture content of <39% (e.g. Cheddar, Parmesan), and vi) processed cheese which includes spreads, cheese foods and slices. The evaluation of these food products took into account many factors such as annual servings, annual consumption, duration of home storage, contamination frequency, growth rate during home storage and contamination level at retail. Based on the data available to the FDA in 2003, the risk assessment stated that soft unripened, soft ripened and semi-soft cheeses had a contamination rate between
2-5%, deemed moderate, while fresh soft cheese, hard cheese and processed cheeses had a low contamination rate (<2%). More recently (2012) a joint risk assessment conducted by FDA and Health Canada (FDA, 2015) concluded that there is a 50-to 160-fold increase in the risk of listeriosis from a serving of soft-ripened raw-milk cheese, compared with cheese made from pasteurized milk. According to the assessment, the predicted concentration of \textit{L. monocytogenes} in soft cheeses is estimated to be $7.6 \times 10^2$ CFU/g for 90% of cheeses made from pasteurized milk, and $2.2 \times 10^3$ CFU/g for 50% of cheese made from raw milk. In addition the assessment further estimates that 10% of raw milk soft cheese will have \textit{L. monocytogenes} concentrations as high as $2.0 \times 10^6$ CFU/g or more. Although the risk from raw-milk cheese is higher, the risk assessment pointed out that pasteurized-milk cheese does carry some risk, as well. It was shown that testing every lot of raw milk cheese for \textit{Listeria} results in a level of safety which is greater than use of pasteurized milk in cheese making.

Wagner at al., (2007) investigated 10 different cheese types for the presence of \textit{L. monocytogenes} including soft smear cheeses and reported that incidence of the pathogen between 3-5%, a result that agrees with the findings of Rudolf and Scherer, (2001). However in Wagner et al., (2007) reported there were no soft smear cheese that were found positive for \textit{L. monocytogenes}. In contrast, Rudolf Scherer, (2001) reported that presence of \textit{L. monocytogenes} in soft cheese, semi soft and hard was 6.3%, 7.6% and 4.4% respectively.

positive for *L. monocytogenes*. Although this study confirmed the potential of producing safe raw milk cheese, their cheese samples were predominantly from hard and semi hard cheeses and washed rind cheeses were not included in their survey. Their data are contradicted by the findings of Little et al., (2008) where an investigation of retail cheese samples for the same pathogens showed that 8.4% of the samples tested had a pathogen present. *L. monocytogenes* was found in two samples of soft cheeses with a concentration of $10^2$ cfu/g which is borderline based on E.U. regulations. Interestingly, if the U.S. standards for presence of *L. monocytogenes* (0 cfu/25g of sample) were applied to the same samples tested by Little et al., (2008) then all raw milk soft cheeses made 806 samples would be deemed contaminated and not fit for human consumption. The study concluded also that unripened raw milk cheeses were more likely to be contaminated while pasteurized semi hard cheeses were most likely to be contaminated from specialist, cut to order, cheese shops

Survival of *L. monocytogenes* in cheese depends on many factors including the type of cheese produced, along with manufacturing, storage and aging conditions. Due to the fact that this pathogen can survive under cold storage temperatures, this makes the control of this bacterium more challenging (Bernini et al., 2013). There are several factors that exist in the cheese matrix that can influence the survival, growth and in some cases the control of *L. monocytogenes*. These factors include the range of pH, sodium chloride content, aw, free fatty acid concentration and competitive microflora. These factors change over time as cheeses age and mature.
1.22 Microorganisms found in cheese rinds

Cheese rinds have a unique microbial composition due to the fact that they are exposed to the environment more than the core of the cheese. An early study by Feurer et al., (2004) investigated the rind microbial diversity of soft red smear cheeses using both cultural and rDNA methods (SSCP polymorphism analysis of the V3 region of 16S rRNA) using both raw milk and pasteurized industrially produced cheeses. The data from this study showed that pasteurized cheese had a more restricted microbial composition while raw milk cheese had a wider microbial distribution. Through cultural methods, pasteurized cheese showed 3 dominant species (Arthrobacter arilaitensis, Carnobacterium maltaromaticum and Lactococcus lactis) which accounted for more than 50% of the total bacterial flora. In contrast, raw milk cheese had a more even distribution of 9 species (Corynebacterium casei, Brevibacterium linens, Marinolactibacillus psychotolerans, Lactobacillus curvatus, Streptococcus thermophilus, Microbacterium gubbeenense, Brahybacterium tyrofermentans, Brevibacterium linens, and Arthrobacter arilaitensis). PCR methods showed a different distribution which emphasized the biases for both methods of analysis. In conclusion, the study showed that there were species that 16S rRNA sequencing was not able to trace, possibly due to preferential amplification or primer design and simultaneously there were species that cultural methods were not able to detect either because the cells were dead (free DNA), live but non-culturable, anaerobic, or inactive cells that require special conditions to grow. The bacterial populations between the two cheeses were very different, with the raw milk cheese having higher diversity and
the study did find that marine bacteria represented a major fraction of the total bacterial populations found on the surface of red smear cheeses.

High throughput sequencing has been used to identify subpopulations of bacteria in cheeses by targeting the V4 region of the 16S rRNA gene (Quigley et al., 2012). These studies showed that there are 19 different genera associated with cheese rinds. The Genera *Corynebacterium, Facklamia, Flavobacterium* and *Cronobacter* were only found in the cheese rind. Smear/wash ripened cheese had low levels of lactococci although overall *Lactococcus* remained the most predominant genus present. In addition *Vibrio* was only detected in smear/washed cheese rinds. Other genera present were *Psychrobacter, Brevibacterium, Leuconostoc, Lactobacillus, Pseudoalteromonas Brachybacterium, Prevotella, Arthrobacter, Streptococcus, Tetragenococcus*, and *Facklamia*.

More recently Wolfe et al. (2014) conducted a large scale *in situ* characterization of microbial communities of cheese rinds. The study first concluded that cheeses aged in different geographical locations do not show significant differences in the rind composition. It was concluded that the factor with the highest effect on the composition of the rind was moisture content. Furthermore, it was found that the composition of the washed rind cheeses showed an uneven distribution of colonization between fungi and bacteria. Of the 24 genera that this study identified in all the samples tested, one of the most abundant microorganisms in bloomy rind cheeses were 4 genera belonging to the phylum *Proteobacteria*. Other major genera present in washed rinds were *Actinobacteria, Firmicutes and Ascomycota*. 

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1.23 Rind development

Feurer et al. (2004) and Millet et al. (2006) emphasized the importance of identifying and understanding the interactions of the cheese rind microbial community of soft ripened cheeses. According to Corsetti et al. (2001) there are two categories of soft ripened cheeses: bacterial smear surface ripened and mold surface-ripened. The first category defines cheeses where bacterial communities are present in large numbers and play a significant role in determining the final characteristics and attributes of the cheese. Some examples of cheese that belong in this category are Tilsit, Limburger, Beaufort and Taleggio (Corsetti et al., 2001). Genera that are usually dominant in these types of cheeses at the end of the ripening period are *Brevibacterium*, *Arthrobacter*, *Micrococcus* and *Corynebacterium*. Smear cheese are characterized by a more rapid ripening process, more intense flavor, small size of the cheese and frequent turning and smearing of the surface of the cheese especially during the early stages of ripening (Corsetti et al., 2001). In contrast, mold ripened cheese, such as Brie or Camembert, depend on organisms like *Penicillium camemberti* (Addis et al., 2001), *Debaryomyces Hansenii* (Lessard et al., 2012) or *Penicillium roqueforti* (Flórez and Mayo, 2006) to rapidly increase the pH of the surface of the cheese, usually within 7 days, and allow bacterial growth (Lessard et al., 2012, Bockelmann et al., 2005), to give the cheese the distinct appearance, aroma and flavor (Corsetti et al., 2001).

Other organisms that play significant role in the development of the rind and the organoleptic properties of soft smear ripened cheeses are: *Candida*, *Cryptococcus*, *Debaryomyces*, *Geotrichum*, *Galactomyces*, *Hansenula*, *Kluyveromyces*, *Pichia*,...
*Rhodotorula, Saccharomyces, Yarrowia, and Zygosaccharomyces* (Addis et al., 2001). Yeast function during aging is mostly seen at the early stages of ripening. They reach their highest number of $10^8$-$10^9$ CFU/g by day 7 during ripening of cheese. Yeast involvement in the ripening process is both direct and indirect. Yeast activities on the surface of the smear cheese includes assimilation of lactate, formation of alkaline metabolites, liberation of bacterial growth factors, fermentation of lactose, lipolysis (Addis et al., 2001), proteolysis, and formation of aroma compounds (Corsetti et al., 2001).

The metabolism of lactate by yeasts and the formation of ammonia from amino acid deamination lead to the de-acidification of the cheese surface enabling the growth of less acid tolerant but more proteolytic and more salt tolerant microorganisms such as *Micrococcus spp.*, *Brevibacterium spp.*, *Arthrobacter sp.*, and *Corynebacterium spp.* (Corsetti et al., 2001; Addis et al., 2001; McSweeney, 2004). Presence of yeasts has also been shown to support the growth of *Enterococcus spp.* and *Staphylococcus spp.* (Addis et al., 2001). The presence of *Yarrowia lypolitica* on the rind was also shown to have weak inhibition of *L. monocytogenes* and *Bacillus cereus* in a study conducted by Addis et al., (2001) on Camembert and blue veined cheeses. Bacterial development in cheeses can also lead to a decrease in reduction of bitterness of the cheese through bacterial enzyme activity. The bacteria produce extracellular proline aminopeptidase that recognizes tri- and oligopeptides that contain a Pro-X sequence at the N-terminus. These peptides can cause bitterness but can be inactivated through hydrolysis by the aminopeptidase enzymes. Indirectly, peptide hydrolysis can contribute to flavor formation and textural changes during ripening (Corsetti et al., 2001).
The function of most of the bacterial species is not yet clearly understood but generalizations can be made at the genus level. Although most of the cheese research has been done on Cheddar cheese, the function of the microorganisms should not be completely different. While the use of starter bacteria is primarily for the production of lactic acid, and LAB cultures are added at concentrations of $10^5$-$10^7$ CFU/ml, non-starter bacteria can be found in various concentrations, ranging from 100CFU/ml to $10^6$ CFU/ml, in artisan cheeses. *Leuconostoc* and *Enterococcus spp.* are usually found in all artisanal cheeses and contribute mainly to flavor development. Also, enterococci have been known to produce antibacterial proteins (enterotoxins) with activity against food-borne pathogens like *L. monocytogenes* and *S. aureus* (Cogan and Sherer, 2007).

Coryneform bacteria are facultatively anaerobic, Gram positive bacteria that are widely distributed in the environment. Their role in rind development is not yet fully understood but they seem to be responsible for color development. Some *Arthrobacter spp.* have been shown to produce clearing zones *in vitro* against *L. monocytogenes* (Carnio et al., 1999), which suggests that they might have an inhibitory effect against pathogens (Fox et al., 2000).

**1.24 Control of *Listeria***

Even though the food products are extensively tested for the presence of *L. monocytogenes* it is very difficult to completely eliminate the bacterium from the processing plant environment (Santorum et al., 2012). Control of *L. monocytogenes* in cheeses is challenging due to the fact that washed rind and other soft cheeses are subjected to minimal processing before packaging, have high moisture content, have 60 days or less
of ripening time, short shelf life and are usually consumed as a “Ready to eat” foods (D’Amico et al., 2008a; Guenthner and Loessner, 2011). About 30% of the major \textit{L. monocytogenes} outbreaks can be traced back to contaminated cheese (Guenthner and Loessner, 2011). D’Amico et al. (2008a) concluded that the 60 day aging requirement does not ensure safety against \textit{L. monocytogenes}, when introduced as a post processing contaminant, for both raw and pasteurized milk soft surface mold ripened cheeses when contamination of cheeses occurs post-processing.

In the case of blue cheeses, the pH range is usually between 4.5 -6.5 with a sodium content of 2.3-7%. Potential contamination in these types of cheeses can occur due to the proteolytic ability of the molds that are introduced during ripening (Bernini et al., 2013). \textit{L. monocytogenes} is usually found on the rind or outer surface of the cheese and can be introduced on the edible portion of this food during slicing. The environment in the rind of cheeses highly favors the survival and growth of \textit{L. monocytogenes}. Of all the cheese types tested, soft cheeses are implicated most frequently as the source of infection in severe outbreaks (Cataldo et al., 2007). It was shown that the pathogenicity of \textit{L. monocytogenes} is directly associated with its acid tolerance which allows it to cross the acid barrier of the stomach as well as to survive high acid conditions during the initial steps of manufacturing (O’Driscoll et al., 1996).

Natural microbes in raw milk exist in great orders of magnitude. Bacterial flora of traditionally produced cheese is expected to be much more diverse and variable when compared with a cheese made from pasteurized milk (Feurer et al., 2004). Advances in molecular technologies allows researchers to use more sensitive methods like
pyrosequencing-based 16S rRNA (Quigley et al., 2012; Wolfe et al., 2014) and Next Generation Sequencing (NGS) (Lusk et al., 2012) that are more sensitive and culture independent. Initial work on cheese rinds and on general cheese communities was limited by cultural methods due to the fact that microbial communities in cheeses have high diversity, low culturability and natural growth environments that are not easily reproducible in laboratory settings (Wolfe et al., 2014). Several studies have previously tried to identify the microbial community in cheeses by using culture based methods which can be biased and labor intensive (Lusk et al., 2012). Other culture independent methods employed were 16S rRNA through denaturing or temperature gradient gel electrophoresis (DGGE/TGGE), restriction fragment length polymorphism (RLFP) (Lusk et al., 2012), terminal restriction fragment length polymorphism (TRLFP) (Brodie et al., 2002) and single stranded conformation polymorphisms (SSCP) (Quigley et al., 2012). Although introduction of molecular methods has led to new insight into bacterial diversity, all of these methods have biases such as specific lysis, preferential amplification and cloning, or formation of chimeric PCR products (Feurer et al., 2004).

The use of starter cultures in cheese making is well documented (Leroy and Vuyst, 2004). Starter cultures have a wide range of antimicrobial metabolites which include organic acids, diacetyl, acetoin, hydrogen peroxide, antibiotics and bacteriocins (O’Sullivan et al., 2002). One group of bacteria that have been investigated for their anti-Listeria properties are the lactic acid bacteria (LAB) (Cleveland et al., 2001). Lactic acid bacteria can either be introduced to the milk especially in cheeses made from pasteurized milk, and can be found naturally as part of the normal flora of the milk in raw milk cheeses.
Lactic acid bacteria are introduced in high numbers as a starter culture to the milk. LAB are characterized by being Gram positive, catalase negative, non-sporeforming, low G+C content, and are non-motile with the exception of *Lactobacillus agilis*, *Lactobacillus ghanensis* (Nielsen et al., 2007) and *Lactobacillus capillatus* (Chao et al., 2008). LAB are designated based on their capacity to ferment sugars primarily into lactic acid via homo- or heterofermentative metabolism (Settanni and Moschetti, 2010). Functions of LAB in cheese making, apart from the initial fermentation and lactic acid production, include curd formation, flavor development and texture (Settanni and Moschetti, 2010) and lastly some, such as *Lactococcus lactis sub. lactis* (Cai et al., 1997), *Lactobacillus plantarum* (Ennahar et al., 1996), and *Lactobacillus delbrueckii* (Miteva et al., 1998) produce anti-microbial substances (bacteriocins) that are active against *L. monocytogenes* (Cleveland et al., 2001).

Bacteriocins from LAB microorganisms usually belong to two classes, class I and class II (Chatterjee et al., 2005; Diep and Nes, 2002). Class I LAB bacteriocins are small (<5 kDa), heat stable peptides that are modified after translation, which results in the formation of thioether amino acids lanthionine (Lan) and methyllanthionine (MeLan) (Chatterjee et al., 2005; Jack and Sahl, 1995). Class II LAB bacteriocins are also small (<10kDa) and heat stable but do not contain Lan residues (Diep and Nes, 2002; O’Sullivan et al., 2002). The mode of action for bacteriocins generally involves interference of the cell wall biosynthesis or causing pore formation in the membrane of the target organisms (O’Sullivan et al., 2002).
Lactic acid bacteria that are associated with food production belong to the genera *Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus and Weissella* (O’Sullivan et al., 2002; Lavanya et al., 2011). LAB are beneficial to the host and they are resistant to antibiotics. In addition, certain strains produce extra cellular polysaccharides (EPS) which assists the cell in adherence and participation in cell to cell interactions. Incorporation of LAB bacteria in dairy foods usually provides viscosifying, stabilizing and water – binding functions (Lavanya et al., 2011).

To date, the only bacteriocins that are commercially available for cheese making are nisin, produced by *Lactococcus lactis sub lactis* and pediocin (PA-1) produced by *Pediococcus acidilactici* (marketed as Nisaplin™). Nisin can be introduced to the food product either by addition of dried concentrated powder during production (E.U. only) or by the use of bacteriocin-producing strains (O’Sullivan et al., 2002). Nisin producing strains have slower rates of acid development and limited proteolytic activity and ability to ferment sucrose. In addition nisin producing strains are more sensitive to bacteriophage (Leroy and Vuyst, 2004). To counter these problems cheesemakers use multiple strains starters some of which are nisin resistant fast acid starters (O’Sullivan et al., 2002).

A major limiting factor for the growth of *L. monocytogenes* on the surface of soft ripened cheeses is an acidic pH level (Guenther and Loessner, 2011). LAB though their fermentation processes discussed earlier are able to rapidly acidify milk through the production of lactic acid (Leroy and Vuyst, 2004). *L. monocytogenes* is not remarkably acid tolerant and cannot grow at pH below 4.5 to 4.6 (Koutsomanis et al., 2003). However
Morgan et al., (2001) showed in his research that *L. monocytogenes* does not behave the same way as other pathogens and it is highly adaptable to environmental cues. In that study *L. monocytogenes* that were exposed to lactic acid conditions were shown to have reduced numbers during the first days of production but were able to be isolated after 42 days. This study concluded that the pathogen was able to adapt and although the CFU counts were reduced, it was never eradicated. *L. monocytogenes* pathogenicity is associated with its acid tolerance which influences its capability to cross the acid barrier of the stomach, survive and multiply. Exposure of *L. monocytogenes* to mild acid conditions, a phenomenon called “stress hardening”, may introduce an acid tolerance response that can increase its ability to survive lethal pH levels (Cataldo et al., 2007). Koutsomanis et al. (2003) in their study showed that a mild acid shock of *L. monocytogenes* strains (serotypes 4b, 1/2a, 3, 1/2b) at pH 5.0, 5.5, and 6.0 increased the ability of the pathogen to survive at the lethal pH of 3.5 when compared with the control groups.

**1.25 Microorganisms with anti-*Listeria* properties other than LAB**

A previous study by Saubusse et al., (2007) used the single strand conformation polymorphism PCR method to identify cheese bacterial communities that have anti-*L. monocytogenes* properties. The study found that lower counts of *L. monocytogenes* were associated with the presence of *Enterococcus faecium, Enterococcus saccharominimus, Chryseobacterium* spp., *Clostridium flavescens, L. garvieae, and L. lactis*. When these isolates were inoculated into pasteurized milk in order to investigate their anti-*Listeria* properties only *L. garvieae, and L. lactis* showed promising results.
*Staphylococcus equorum* was investigated for its anti-*Listeria* properties (Goerges et al., 2006). In 1999, Carnio et al. reported a new bacteriocin produced by *S. equorum*, micrococcin P₁, a high molecular weight bacteriocin. *S. equorum* was revealed to be a potent inhibitor of growth of *L. monocytogenes* on the cheese surface of soft cheeses. *Brevibacterium linens*, a strain typically used in the production of red smear cheese, also produces a bacteriocin, linocin M18, which acts against *L. monocytogenes* (Goerges et al., 2006). Other microorganisms that have shown potential anti-listerial properties are yeasts (Goerges et al., 2006). Earlier studies by Dieulevux et al. (1998) were able to isolate two inhibitory compounds, D-3-phenullactic and D-3-indollactic acids, produced by *Geotrichum candidum*. Goerges et al. (2006) showed that *Candida intermedia* was able to achieve a 3 log reduction of *L. monocytogenes*.

1.26 Bacteriophages

Bacteriophages are the most abundant microorganisms on Earth (10^{31} particles) that are widely spread and classified into 13 families based on their shape, size type of nucleic acid and presence/absence of envelop or lipids in their structure (Garcia et al., 2010). Most of the phages belong to the *Caudovirales* order and are classified into 3 families based on the morphological structure of their tail. These are: *Myoviridae* (contractile tail), *Siphoviridae* (long non contractile tail), and *Podoviridae* (extremely short tail) (Garcia et al., 2010). Phages can also be distinguished based on their life cycle, between lytic and temperate, with the difference being that the lytic life cycle leads to bacterial cell lysis while the latter can incorporate its DNA into the bacterial DNA but may enter a lytic cycle.
due to environmental signals (e.g. cells stress due to the presence of antibiotics) (Garcia et al., 2010).

Bacteriophages are viruses that invade bacterial cells and in the case of lytic phages disrupt bacterial metabolism and cause the bacterium to lyse (Garcia et al. 2008). Recently there are two commercial products that have been approved by the FDA (FDA 2006) for use in ready to eat foods: *Listex* (P100), which consists of two phage cocktails against *L. monocytogenes*, and *LMP 102*. In 2007, OmniLytics Inc. received FDA approval for an anti-*E. coli* and an anti-*Salmonella* phage-based product to treat live animals prior to slaughtering (Garcia et al., 2010). Bacteriophages were first discovered by Earnest Hankin (1896) and Frederick Twort (1915) but were used as a treatment for dysentery for the first time by Felix d’Herell (1919) (Garcia et al., 2008). Bacteriophages are usually regarded as natural enemies of bacteria that attack live cells, generally do not cross species and genus boundaries, do not affect starter cultures and commensals, and are not classified as xenobiotics (Carlton et al., 2005). Bacteriophages can be used at all stages of production in the classic ‘farm to fork’ approach either to prevent or reduce colonization, decontamination of raw products, contact surfaces and equipment and to extent shelf life of perishable foods (Garcia et al., 2008). Most of the phages that infect organisms of the genus *Listeria* are temperate and feature a very narrow host range (Carlton et al., 2005).

Guenther and Loessner (2011) investigated the efficacy of bacteriophage A511, a broad host range phage, against *L. monocytogenes* on the surface of white mold ripened cheeses (Camembert-type) and a washed rind cheese with a red-smear culture (Limburger-type). Their findings suggested that application of phage A511 is able to eradicate *L.
cells from the surface of the cheeses tested to levels below 1cfu/g when contamination at the beginning of the ripening phase was at 100cfu/cm² or below. At higher levels (10³cfu/cm²) a 3 log reduction was observed. The study also showed that the efficacy of the phage depends on the type of soft cheese produced. More specifically infectivity of the phage was less affected in red smear cheeses than in white mold ripened cheeses. Most likely this is because the high proteolytic activity of the surface starter and ripening cultures affects infectivity and integrity of the phage (Guenther and Loessner, 2011).

Use of bacteriophages for control of L. monocytogenes in soft ripened cheeses as well as other RTE foods should take in account several factors. Application of the phage is usually required at high levels (10⁸-10⁹ pfu/cm²) (Guenther and Loessner, 2011). Timing and frequency of application are also very important (Silva et al., 2014). Phages must be introduced to the cheese surface precisely at the time that corresponds with the possible time point(s) of Listeria entry to the product (Guenther and Loessner, 2011). Late application of the phage tends to reduce the efficacy of the phage due to the increased microbial population and diversity on the surface of soft cheeses (Guenther and Loessner, 2011). Phages should be investigated for the presence of genes that code for toxins prior to their application in the food industry, as this will have negative effects for the consumer (Strauch et al., 2007). Lastly like every other biological control method the risk for emergence of resistance exists (Guenther et al., 2009), however currently none of the strains tested against phage A511 showed any indication of this trend (Guenther and Loessner, 2011). Apart for the use of bacteriophages directly in cheese as a control method, phages can also be used in the machinery and environmental areas of production to either
assist in sanitation of food surfaces or to remove *L. monocytogenes* biofilms (Soni et al., 2010).

1.27 Non biological control methods

Linton et al., (2008) investigated the potential of controlling *L. monocytogenes* in raw milk using hydrostatic pressure. The milk that was used for this study was bovine and was inoculated with 2 or 4 log cfu/ml *L. monocytogenes*. After treatment with 500MPa for 10 min at 20˚C prior to making Camembert style cheese, the authors reported that *L. monocytogenes* was below detectable levels in the pressure treated milk, curds and final cheese product indicating the potential of this method for raw milk cheese making. Effective environmental samples in collaboration with a defined corrective actions to be implemented for positive findings can also help in the control of this pathogen (Tompkin, 2002).

1.28 Regulations of significance to this research

Approximately 10 billion pounds of cheese per year are consumed by Americans according to a United States Department of Agriculture 2012 report (Gould et al., 2014). In the U.S., there is currently zero tolerance as the regulatory limit for *L. monocytogenes* in ready to eat foods, which is 0 CFU/ in 25g or ml of food (Gasanov et. al, 2005). The European Union currently has two regulatory standards for *Listeria*, one applied to foods intended for infants or individuals with compromised immune system; which is identical to the U.S. regulation (0 CFU/25 g of food). The second regulation allows for the presence of 100 CFU/ 25g of food during the shelf life of the product and for foods intended for consumption by healthy individuals and non-infants. In Australia, the presence of 10cfu or
more in 25g of ready to eat (RTE) food previously implicated in human listeriosis or 100 cfu/g in other packaged RTE foods can lead to a recall (Jadhav et al., 2012).

The European Food Safety Authority (EFSA) survey of presence of *L. monocytogenes* in cheese samples showed that the incidence of the pathogen was 0.47% for the time period 2010-2011 (EFSA, 2013). The incidence of *L. monocytogenes* reported by EFSA seems to agree with the findings of Lambertz et al., (2012) where the authors reported that in cheese samples from Sweden, incidence of the pathogen was 0.4%. For the year 2011 there were 1476 confirmed listeriosis cases in the E.U. with 93.6% of those cases hospitalized, with a mortality rate of 12.7% (134 deaths). In comparison there approximately 1600 illnesses and 260 deaths due to listeriosis that occur annually in the U.S. (Scallan et al., 2011).

The World Health Organization (WHO) reported that, on average, there are 1.7 billion cases of diarrhea each year from infection caused by bacteria, viruses or parasitic organisms, malnutrition, contaminated water or poor personal hygiene. Diarrheal disease is the second leading cause of death in children under 5 years of age and is responsible for killing approximately 760,000 children every year. WHO’s (2014) on foodborne illnesses and food safety estimated that food sources contaminated with bacteria, parasites, viruses or chemical substances are the cause of 2 million deaths each year (Bergholz et al. 2014). In parallel with increased world population size, consumer demand for a wider variety of foods is also increasing, which makes the food supply chain longer and more complex (WHO, 2015). The report emphasizes the need for good collaboration between governments, producers and consumers since the food supply chains regularly cross
multiple borders. Due to the emerging need for higher standards for food safety many organizations, including WHO, United Nations Food and Agriculture Organization (FAO), World Health Assembly (WHA), The U.S. Food and Drug Administration (FDA), and the European Union (E.U.), have initiated programs to increase food safety (Luber, 2011). WHO in collaboration with FAO has taken a central role in developing guidelines to strengthen and harmonize food safety, through the joint managed Codex Alimentarius Commission (CODEX). In 2010, the 63rd WHA adopted a resolution (WHA Resolution 63.3, Geneva, 2010) forming a strategic plan that requires the WHO to: (i) provide the evidence base for measures to decrease food borne risks along the entire food chain, (ii) improve international and national cross-sectoral collaboration, and (iii) provide leadership and assist in the development and strengthening of risk based, integrated national systems for food safety. In addition to the strategic plan, WHA, through a partnership between WHO and FAO has instituted a global system, The International Food Safety Authorities Network (INFOSAN), which can rapidly share information during food safety emergencies (WHO 2014).

In the United States (US) the FDA Food Safety and Modernization Act (FSMA) was signed into law by President Obama on January 4th 2011. FSMA regulations will go in effect beginning January 2016. The aim of this sweeping reform is to ensure that the U.S. food supply is safe by shifting the focus from responding to contamination to preventing it (FDA, 2011). The U.S. Centers for Disease Control and Prevention (CDC) estimates that 46 million people (1 in 6 Americans) get sick each year with 128,000 hospitalizations and 3000 deaths from foodborne diseases (FDA, 2015; Scallan et al.,
FSMA regulation mandates the need for preventative controls for food facilities, mandates rules for produce safety, establishes preventative controls for animal feeds and requires prevention of intentional contamination of food. The law also mandates increased inspection frequency based on risk, production and safety record access, and requires food testing from accredited laboratories. Others areas of the law include the ability of the FDA to issue a mandatory recall, detention of product if there is a potential violation of law, suspension of registration, creation of a system for effective tracking of domestic and imported food, and guidelines may be proposed for additional record keeping for high risk foods. For imports, the law gives FDA the authority to deny entry of a food, requires importer responsibility to ensure that all suppliers have adequate controls to ensure food safety, and requires third party certification with the ability to request certification for high risk foods as a condition of entry (FDA, 2015). However the law specifies that to implement these new rules, the agency will rely on food inspectors from other agencies (Federal, State or local) to meet this increased inspection mandate.

CDC reported that in 2013 there were a total of 818 reported food borne outbreaks with the most common causative agents being norovirus (35%) and Salmonella (34%). The outbreaks resulted in 13,360 illnesses, 1,062 hospitalizations, 16 deaths and 14 food recalls. Dairy outbreaks accounted for 10% of the outbreaks with 3 deaths caused by L. monocytogenes. Although the CDC states that the food vehicle for the Listeria outbreaks was cheese, it neglected to point out that the cheese was consumed at a private home and that the cheeses were mostly Latin style, Le Frére or Mexican soft cheese. In only one of those outbreaks was the cheese was made from pasteurized milk (FOOD, 2015). Gould et
al. (2014) reported that between 1998 and 2011 approximately 40% of the outbreaks caused by cheese were linked to soft cheese imported from Mexico. The study also concluded that unpasteurized milk was most likely contaminated at the point of processing typically through infected animals or the environment. This conclusion solidifies the importance of animal husbandry and environmental monitoring for identification of \textit{L. monocytogenes} presence and application of control strategies. In 2007, CDC through sampling studies, reported that the incidence of \textit{L. monocytogenes} in food samples was shown to be approximately 11% (Lecuit et al., 2007). \textit{L. monocytogenes} is a pathogen that can survive in various environments ranging from highly acidic to high salt and within a broad range of temperatures (Ferreira et al., 2011)

As FSMA rules begin implementation, the notorious foodborne pathogen \textit{L. monocytogenes} will no doubt impact produce safety rules, rules for animal feed and regulations concerning cheeses made by raw milk. The following chapters will address these important issues in an ever changing regulatory landscape.
CHAPTER 2: THE IMPACT OF A SOFT WASHED RIND CHEESE MICROBIAL COMMUNITY ON GROWTH AND DETECTION OF *LISTERIA MONOCYTOGENES*. 
2.1 Introduction

Listeria monocytogenes is a Gram positive rod-shaped facultatively anaerobic bacterium that is a major foodborne pathogen. The bacterium has been found in various foods such as meats, vegetables and dairy products (Ryu et al., 2013; Ryser et al., 1997). Raw milk cheeses have been identified as high risk products for transmission of this pathogen (Millet et al., 2006). This is especially true for washed rind cheeses due to the procedures used during manufacture that increase risk for accidental introduction of the pathogen. Guenther and Loessner (2011) reported that approximately 30% of all major L. monocytogenes outbreaks have been traced back to contaminated cheeses. Gould et al. (2014) recorded 90 outbreaks where cheese was the implicated food that were reported to
the CDC between the years 1998-2011, which resulted 1882 illnesses, 23 hospitalizations and 6 deaths. The pasteurization status of the milk used to make the cheese was reported for 82 (91%) of the outbreaks: 38 (46%) were caused by cheese made from unpasteurized milk and 44 (56%) were caused by cheese made from pasteurized milk. The incidence of *L. monocytogenes* contamination frequency in cheese ranges from 1% to 22%. Soft and soft ripened cheeses are more frequently contaminated due to the fact that they provide appropriate growth conditions for *Listeria* (Ryser and Marth, 1989; Back et al., 1993; Rudolf and Scherer, 2001; Guenther and Loessner, 2011). The increased demand for raw milk artisan cheeses by consumers seeking the organoleptic characteristics and the texture that raw milk cheeses can deliver has the potential to increase consumer exposure to *Listeria* unless risk reduction practices are employed during production (Pintado et al., 2005; D’Amico et al., 2008a). There are approximately 1600 cases of listeriosis reported in the United States annually and of these, 260 cases are fatal (Scallan et al., 2011). This pathogen is more dangerous to immunocompromised hosts, newborns, pregnant women and the elderly population which makes the control of this bacterium an important public health issue. For healthy individuals, a high inoculum is typically required (10^5 to 10^6 CFU/g) to cause the disease (Maijala et al., 2001). However for susceptible individuals a concentration of 100 CFU/g or lower may be sufficient to cause listeriosis (Sip et al., 2012).

Cheeses within the soft washed rind category include notable varieties such as Reblochon de Savoie (Mariani et al., 2007), Limberger, Tallegio (Eppert et al., 1997) and Vacherin Mont d’Or (Carnio et al., 1999; Beresford et al., 2001). There are various ways in which *Listeria* can be introduced to a soft washed rind cheese. It can be introduced
through use of contaminated milk, equipment, or post production contamination through human activities or through contact with the processing environment. The bacterium itself has the ability to survive under conditions of low pH, low temperature and high salt concentration (Ryu et al., 2013; Guenther and Loessner, 2011). Soft washed rind cheeses such as Limburger are defined by the Food and Drug Administration (FDA) as cheeses that can be made from unpasteurized milk if they are ripened (aged) at a temperature of no less than 35°F (1.6°C) for no less than 60 days (Gould et al., 2014; USFDA, 2003). The first step in the manufacture of these types of cheeses is the inoculation of the milk with starter cultures (Irlinger and Mounier, 2009), then 30 min after rennet addition the curd is cut and gently stirred to eliminate whey. The curd is then molded, pressed, and salted in brine. The cheeses are then smeared (washed) regularly during the early stages of ripening. Cheeses are kept at 95% relative humidity and at temperatures ranging from 13°C to 17°C (Mariani et al., 2007). One of the most significant periods of cheese production is the ripening process during which time there is a high activity of starter cultures, non-starter cultures, indigenous milk enzymes and chymosin that work towards the development of the organoleptic and textural properties of the cheese (Brennan, 2002). Previous studies have shown that *Listeria* can survive on the rind of the cheese and its incidence on the rind was positively correlated with long term storage (Bernini et al., 2013). Previous research has focused on elimination of *Listeria* through use of nisin-producing starter cultures (Abee et al., 1994), and LAB starter cultures and bacteriophages (Guenther and Loessner, 2011). Only recently has inquiry been devoted on investigation of the role of the natural milk microflora on *Listeria* ecology in cheese. The task of understanding how microbial
communities form in cheese and on cheese surfaces, or what organisms predominate on the cheese during ripening, was previously very difficult due to high species diversity, low culturability and inability to re-create their natural microbial environment. Recent advances in high-throughput sequencing have allowed researchers to investigate microbial communities and to try to reconstruct these communities in the lab. In a recent study, Wolfe et al., (2014) examined the microbial composition of cheese rinds from 137 different cheese types from both Europe and the United States. Their findings showed that there are 14 bacterial and 10 fungal genera that are found at greater than 1% average abundance in those communities. Surprisingly, the composition of the microbial communities was not significantly correlated with geographical origin but it did correlate strongly with surface moisture.

Apart from pathogens, cheese can be exposed to spoilage organisms, one of the most common being Pseudomonas spp. These are aerobic, non-spore forming Gram negative rod-shaped bacteria that can produce distinguishable colony morphologies or pigmentation (i.e. blue green or yellow green fluorescent pigments). Psychrotolerant Pseudomonas spp. are characterized as major food spoilage organisms (Martin et al., 2011) mainly due to their extracellular enzymes: lipases, proteases and lecithinase (Arslan et al., 2011). Contamination from these microorganisms causes undesirable changes in appearance, texture, flavor, and odor that reduces food quality (Martin et al., 2011).

The use of molecular methods is highly desirable since it can provide comprehensive information about the cheese microbial community, allowing observation of the shift in population dynamics during the ripening process in real time. In addition,
observations of behavior of the cheese microbial community in the presence of a pathogen can be made. In the present study, we introduced a cocktail of 6 different strains of *L. monocytogenes* to the surface of a soft washed rind cheese to investigate if the pathogen affected the composition of the cheese rind microbial community, and whether the indigenous cheese microbial community has any effect on the survival and growth of the pathogen. This study also investigated the presence of a common spoilage organism, *Pseudomonas spp.* and how its activity compared with the behavior of *Listeria spp.* during the early stages of cheese ripening.

2.2 Methods

2.2.1 *Listeria* Challenge Cultures

Unripened samples of an experimental soft washed rind raw milk cheese, obtained from a local cheese maker, were inoculated with a cocktail comprised of 6 different strains (Table 1) of *L. monocytogenes*, which were obtained from raw milk, artisan cheese or their respective processing environments.

Stock cultures of *L. monocytogenes* previously isolated, ribotyped, and frozen at -80°C were used to inoculate the un-ripened samples of experimental cheeses. To prepare the frozen stock cultures for use in the lab, the cultures were inoculated into 9ml of trypticase soy broth with 0.6% yeast extract added (TSYBE) and incubated at 32°C±1°C for 24±2hr with 2 subsequent transfers before use. After serial dilution, Petrifilm™ AC films (3M Microbiology, St. Paul MN) were used to perform aerobic plate count (APC) to determine the viable *L. monocytogenes* counts from the suspensions. Petrifilm™ AC films
were incubated at 32°±1°C for 48 ± 2hr. Based on the results of the APC analysis, equal proportions of cells from each culture were combined into a cocktail yielding 2 X 10^9 cells/ml.

2.2.2 Cheese trials and treatments

Two trials were conducted for this study. Both trials were set up as follows. Three groups of 10 un-ripened two day old brined cheeses were inoculated with the 6 strain cocktail of *L. monocytogenes* at 100 cfu/cm² (LIC) and one group of 10 un-ripened cheeses were kept un-inoculated as controls (UC). Each group of 10 cheeses were placed in separate, sterile incubators to prevent cross contamination. For the first trial cheese wheels were contaminated from the initial brine with *Pseudomonas* and the trial had to be terminated at day 28. Samples from the UC group of that trial were collected until day 28 and are referred further as *Listeria* un-inoculated with *Pseudomonas* (PC). For the second trial the same format was used but the cheesemaker’s aging facility was visited and rind samples, further referred as cheesemaker control (CC) samples, from the same batch as the experimental cheeses were collected on days 16, 31, 37, 43, 52, and 57 (Figure 1).

2.2.3 Cheese ripening conditions

Wheels of the experimental cheeses were produced at a commercial facility. During the production of the experimental cheeses, the commercial facility washed the cheese surfaces in beer and wrapped them in Spruce tree bark to conform to the standards of identity used when producing certain soft-ripened raw milk cheese varieties. Before leaving the commercial facility, the day old experimental cheeses were washed once in a brine solution consisting of 3% NaCl and .5 Danisco Culture Units (DCU) each of
Rhodosporidium infirmominiatum (R2R) and Geotrichum candidum (GEO 15) (Danisco, Denmark) per 1000ml of sterile deionized water. Growth of GEO 15 (as identified as a chalky white surface growth) de-acidifies the surface of the cheese during the first 7 days of ripening. Then R2R yeast grows in response to the lower acidity levels and creates brick red surface growth that is the signature color of the non-experimental cheeses.

After the initial brine wash, the cheeses were aseptically transferred to lab scale cheese aging chambers at the University of Vermont. Upon receipt, cheeses were transferred to aging racks sanitized with 70% isopropyl alcohol. The drying and ripening of the cheese was conducted in a lab scale aging chamber constructed using a modified incubator (Model WC 491BG, Avanti. Miami, FL and Model DWC350BLPA, Danby Product LTD) as described previously (D’Amico & Donnelly, 2008). Cheeses were held at 13°± 2°C with 92 ± 2% relative humidity through day 70.

2.2.4 Pathogen and smear culture application

Upon receipt, three groups of 10 wheels of cheese were inoculated with a 6 strain cocktail of L. monocytogenes (LIC), and 10 wheels of un-inoculated experimental cheese served as controls (UC) (Figure 1). The pathogen inoculation procedure was as follows: the cocktail of L. monocytogenes was serially diluted and inoculated into the wash solution, 3%NaCl and .5 DCU each of R2R and GEO 15 (Danisco, Denmark) per 1000ml of sterile deionized water, in order to obtain a Listeria inoculum of ~3 log cfu/ml of smear solution to provide for cheese contamination levels of 100 cfu/cm². This level was chosen to mimic the European Union Food Safety Objective of <100 L. monocytogenes at the time of consumption (Jadhav et al., 2012). The cheeses were removed from the aging chamber and
each side of the cheese was smeared with 1ml of *L. monocytogenes*-contaminated wash solution using a sterile L-spreader. Control (UC) cheeses were inoculated with 1ml of the uninoculated wash solution and spread over the cheese surface with a sterile L-spreader. The cheeses were washed daily, with alternate surfaces being washed on alternate days, with a sterile surgical sponge for 2 weeks (days 2-14) until surface growth began. After the appearance of surface growth the cheeses were turned daily until day 50 of ripening at which point the cheeses were wrapped in parchment paper and placed back in the aging chamber with no added humidity following the procedures of the commercial manufacturer.

2.2.5 Bacterial enumeration

The following cheese samples were analyzed: cheese upon receipt, after pathogen inoculation (day 1) and during ripening on days 4, 7, 14, 21, 28, 35, 42, 49, 56, 63, and 70. The experimental cheeses were aged for 60-days in accordance with the U.S. Code of Federal Regulations, Part 133 standards for soft cheeses made from raw milk (USFDA, 2003). The experimental cheese samples were analyzed to day 70 to include growth times during distribution and retail. Wedge samples (25g) were taken from the experimental cheese using a sterile knife. The samples were then diluted in 225ml of sterile Butterfield phosphate buffer (a 1:10 ratio). After dilution the samples were stomached for 1 min in a Stomacher® 400 circulator. The resulting homogenate was serially diluted to obtain levels of *L. monocytogenes* that could be counted on CHROMagar™ Listeria (DRG International, Springfield NJ). The resulting homogenate represented $10^0$ and the next dilution of $10^{-1}$ was 1/10 the concentration of the homogenate, and dilution was repeated until a countable
number (25-250) of colonies was achieved. CHROMagar™ Listeria plates were used to isolate \emph{L. monocytogenes} colonies. CHROMagar™ is a chromogenic media that has been developed for faster identification of \emph{L. monocytogenes} (Ehsan et al., 2010). The media employs the presence of phosphatidylinositol phospholipase C (PI-PLC), which is encoded by the virulence gene \textit{plc A}. The activity of this enzyme produces a white halo around the hemolytic \emph{L. monocytogenes} and \emph{L. ivanovii} colonies (Ehsan et al., 2010; Zunabovic et al., 2011). The activity of the enzyme PI-PLC was combined with chromogenic substrate for \(\beta\)-D-glucosidase which produces turquoise colonies for all \emph{Listeria} species (Ehsan et al., 2010). The plates were incubated for 48±2h at 37±1ºC. After the incubation period turquoise colonies surrounded by a white halo (indicative of \emph{L. monocytogenes}) were enumerated. For the control experimental cheeses (and instances where the above procedure did not produce detectable colonies of \emph{L. monocytogenes}) 25 g wedge samples of cheese were enriched with Buffered Listeria Enrichment Broth (BLEB) and incubated for 4h at 30±1ºC at which point selective agents (acriflavin, nalidixic, and cycloheximide) were added and incubation continued for 20±1 more hours. After the 24±1h incubation of the primary enrichment, 0.1ml of primary enrichment was added to 10ml of 3-N-Morpholinepropanesulfonic Acid Buffered Listeria Enrichment Broth (MOPS-BLEB) and incubated at 35±1ºC for 24±1h. After incubation, 0.5ml of MOPS-BLEB was screened for \emph{Listeria} spp. using the Genus \emph{Listeria} and \emph{Listeria monocytogenes} Polymerase Chain Reaction (PCR) assay for the DuPont Qualicon BAX Q7 (Wilmington, DE). The PCR assay recognizes specific \emph{Listeria} spp. isolates.
2.2.6 Rind Sample collection and extraction of genomic DNA

Each rind sample was collected using a previously unused and sterilized razor blade. The cheese wheel was selected randomly and a 2x2 cm$^2$ area was scraped. The rind collected was placed in a 1.8ml Eppendorf tube and was stored at -80 °C until extraction. Randomly throughout the duration of this study the cheesemaker’s cheese aging facility was visited and rind samples (CC) from the same batch of the experimental cheese were collected to have as a comparison to the cheese from the same batch that was aging in the lab. This was done in order to ensure that the UC lab samples had similar rind microbial communities as the CC samples in the actual cheese aging facility.

Genomic DNA was extracted using the PowerSoil® DNA Isolation kit (Mo Bio Laboratories Inc., Carlsbad CA) according to the manufacturer’s guidelines. The kit was chosen over other commercially available products due to its ease of operation and the high quality of DNA recovered as observed by Quigley et al. (2012). Extracted DNA was measured using the NanoDrop 2000c (Thermo Scientific™) to obtain ng/µl concentration for each sample. The samples were kept at -20°C for 7 days after which they were transferred to the laboratory of Dr. Rachel Dutton, Harvard University, Cambridge, MA for shotgun sequencing. Bacterial and fungal amplicon libraries were prepared by amplifying the V4 region of 16s rRNA and ITS, respectively, as previously described by Wolfe et al. (2014).

2.2.7 Data analysis

Amplicon data were analyzed through the Quantitative Insights Into Microbial Ecology (QIIME) program (Carporaso et al., 2010). The data sets were subsampled so that
each sample is represented by the same number of sequences or reads. This subsampling produced a 19,500 reads/sampling-day data set for the 16S rRNA and a 1,699 reads/sampling-day data set for the ITS. Beta diversity analysis, which computes the degree of similarity between samples, was computed. Principal component analysis was computed in the 16S rRNA data set as it was more diverse. Further analysis of the data was done using SPSS version 22.0 software (SPSS Inc., Chicago, IL). Multivariate analysis of variance (MANOVA) was calculated between sampling date, control and treatment percentage operational taxonomic unit (OTU) for the *L. monocytogenes* trial 16S bacterial and ITS data sets. The *Pseudomonas* trial data were analyzed in the same manner between sampling date and treatment. The difference between the two trials was that in the former, the initial CFU/ml for the pathogen was known while in the latter *Pseudomonas* was unintentionally introduced by the cheesemaker precluding determination of the initial concentration of *Pseudomonas*. Data are presented graphically for each Genus tested.

### 2.3 Results

Analysis of cheese rinds from all cheese samples shows a greater diversity of bacterial versus fungal species. During the ripening process there were 15 different bacterial genera that were dominant (average abundance >1%) in the cheese rind. The bacterial genera are spread among 3 phyla. These are *Firmicutes* with 6 genera (*Faclamia, Lactococcus, Staphylococcus, Streprococcus, Pseudomonas, and Vagococcus*), *Proteobacteria* with 6 genera (*Yersinia, Morganella, Halomonas, Providencia, Alcaligenes, and Vibrio*) and *Actinobacteria* with 3 genera (*Arthrobacter, Leucobacter, and Micobacterium*) (Table 2). There were only four abundant fungal genera identified by
our ITS analysis, all belonging to the phylum Ascomycota. These were *Galactomyces* (*Saccharomycetales*), *Debaryomyces* (*Saccharomycetales*), *Dibodascus* (*Saccharomycetales*) and *Scopulariopsis* (*Microascales*). Principal component analysis of the 16S rRNA data set for both LC and PC trials showed that the “caves” used in laboratory trials did not introduce bias in the aging of the cheeses based on the sequence analysis from each sample point (Figure 2). The analysis also showed that trial 1 and trial 2 were different as the samples from each trial clustered separately (Figure 3), suggesting parameters which yielded a different composition of the cheese microbial communities. Apart from the obvious fact that there were different bacterial adulterants (*Listeria* versus *Pseudomonas*) this might yield a significant difference in the composition of microbial communities in the rind at the genus level. Trial 2 analysis showed that, overall, there was no difference between the control and *L. monocytogenes* inoculated samples when the microbial communities were compared as a whole. This suggested that there might be differences in the Genus level that this analysis is not able to detect.

**2.3.1 *Listeria* inoculated (LC) samples (Trial 2)**

Figure 4 shows the estimated marginal means derived from the average abundance of each sample of the 15 dominant genera present in the cheeses that were inoculated with the *Listeria* spp. cocktail plotted against sampling day for each. The statistical analysis showed that there were significant differences between the average abundance means for two of the 15 genera identified. These were *Lactococcus* (Figure 4, Panel C, \( p=0.017 \), Wilks \( \lambda=0.011 \)) and *Facklamia* (Figure 4, Panel J, \( p=0.002 \), Wilks \( \lambda=0.011 \)). *Lactococcus*
spp. average abundance was higher at day 7 for the control cheeses (31.1%), compared to 15.5% average abundance in inoculated cheeses at the same sampling day. By day 14, both treatments showed that the presence of *Lactococcus* in the rind was reduced to almost 0% average abundance and only started increasing past day 49. At the end of the trial (day 63), the UC cheeses showed an average abundance of *Lactococcus* at 3%, while LC cheeses had an average abundance of 1%. *Facklamia* data showed that the genus was significantly present in the UC samples ranging from 0.42% average abundance at day 7 to 17.1% average abundance at day 42. Presence of *Facklamia* in the LC samples ranged from 0.75% average abundance at day 7 to 5.2% average abundance at day 56. At day 63 both LC and UC samples had identical measured averaged abundance (4.3%) for *Facklamia*.

The analysis did not find any significant effects resulting from the introduction of 100 cfu/cm² of *L. monocytogenes* on *Yersinia* spp., *Leucobacter* spp., *Pseudomonas* spp., *Morganella* spp., *Vagococcus* spp., *Providencia* spp., *Alcaligenes* spp., and *Vibrio* spp. There were significant main effects for *Halomonas* spp., *Staphylococcus* spp., *Microbacterium* spp., and *Streptococcus* spp. In contrast *Arthrobacter* spp. did not have any significant main effects however the two way interaction between day and inoculum was significant at the 0.1 level (p=0.056) indicating a possible trend. *Halomonas* spp. and *Microbacterium* spp. were significant for both sample day and inoculum of *Listeria*. *Staphylococcus* spp. and *Streptococcus* spp. mean value difference was significant for only sample day and not for presence of *Listeria*. The results do suggest that the presence of *Listeria* can interfere with the average abundance of the normal microflora of the rind but
overall the difference should not produce a significantly different microbial population. No other genus tested had any significant difference observed through this analysis.

Presence of *Listeria* in the rind does seem to change the overall composition of the microbial community. For UC cheeses analyzed on day 63 the most abundant genera present was *Halomonas* spp. (30.5%) followed by *Yersinia* spp. (29.3%), *Microbacterium* spp. (9.3%), *Facklamia* spp. (6.8%) *Staphylococcus* spp. (5.1%), and *Lactococcus* spp. (3.9%). In contrast for the LC samples, *Yersinia* spp. (33.4%) was the most abundant genus followed by *Halomonas* spp. (20.4%), *Leucobacter* spp. (7.1%), *Morganella* spp. (6.1%), *Microbacterium* spp. (4.4%) and *Staphylococcus* spp. (3.9%). All other genera were found below 3% overall presence (Table 2).

*L. monocytogenes*, presence on the surface of the cheese was not identified through our molecular analysis as being among the top 15 predominant genera. Direct enumeration showed that presence of *L. monocytogenes* was sustained for the duration of the study (day 63). *L. monocytogenes* growth increased in the first 14 days post inoculation reaching levels of $10^6$ cfu/g, which were sustained for the duration of aging (Figure 5).

To investigate further this shift in microbial populations, the data were plotted over time and separated by phyla. Figures 6, 7 and 8 show the change in the 3 phyla represented on the surface of the rind in the presence and absence of *Listeria*. For the phylum *Firmicutes* (Figure 6), we observed very little change between samples from cheeses with and without inoculated *Listeria*. All of the microorganisms tend to reduce their presence by day 14 regardless of the treatment to below 1.1%. However UC samples show an increase in *Facklamia* sp. from 1.1% to 17% by day 42 which is not seen in the LC samples.
*Pseudomonas* spp. also shows an increased presence in the LC samples, reaching 6.5% presence by day 49, which is not seen in the control cheeses.

Members of the phyla *Proteobacteria* (Figure 7) and *Actinobacteria* (Figure 8) showed the greatest differences between UC and LC treatments. *Yersinia* spp. was more prevalent in LC samples than UC samples. Although UC samples showed a rapid increase in *Yersinia* between day 7 (10% average abundance) and day 14 (49% average abundance), its growth was not able to be sustained as well as in the LC cheeses. On day 42 both treatments had a presence of *Yersinia* on the rind ranging from 13% average abundance in LC cheeses to 17.5% average abundance in UC cheeses. For the last three sampling dates *Yersinia* in UC cheeses showed a constant presence (ranging from 32% at day 49 to 27.4% average abundance at day 63) while the UC cheeses showed a constant increase from 19.7% at day 49 to 35% average abundance by day 63. Although *Yersinia* abundance on the rind of UC and LC samples behaved differently during the ripening process both, UC and LC treatments, had the lowest abundance for this genus at day 42 (17.5% for UC samples, 13.9% for LC samples).

On the other hand *Halomonas* spp. were more prominent in UC cheeses than LC. Excluding day 42 which is the only sample point that LC samples had a higher presence than UC (31% vs. 25.5%) at all other sampling points the presence of this genus in UC cheeses was higher (Figure 7). *Vibrio* spp. was more prominent in the LC cheeses on day 14 reaching 19% average abundance, however from day 21 the presence of this genus was almost identical to the control cheeses for the duration of the aging process. *Morganella* spp. shows a constant increase in the LC samples when compared to the UC samples for
the duration of the study. Its presence in the LC samples ranges from 3.1% at day 14 to 11.3% at day 49, down to 7.8 at day 63. In comparison in the UC samples Morganella reached a high value of 4.5% at day 56. Lastly for this phylum, Providencia spp. presence in the UC samples was higher than in the LC samples on most sampling days. Providencia in the UC samples reached its highest value on day 49 (5.4% average abundance) while in the LC samples the highest value reached was at day 63 (3.1%). In contrast the average abundance of Providencia at day 63 in the UC cheeses was 3.8%.

The phylum Actinobacteria only had three members however Leucobacter sp. and Microbacterium spp. showed different growth profiles between UC and LC treatments (Figure 8). For the UC samples Microbacterium presence was higher at day 21 (7.7%) and from day 35 (24%) until the end of the aging period (day 63, 7.7% average abundance). In comparison Microbacterium highest abundance in LC samples was seen at day 28 (9.5%) and by day 63 it was reduced to 3.7%. Leucobacter spp. was more prominent in LC cheeses and its presence increased from day 21 (2.9%) reaching its highest value on day 42 (15.1%). In contrast Leucobacter spp. did not significantly increase in the UC cheeses until day 56 (5.8%). Even with this difference both cheese treatments had similar presence of Leucobacter by day 63 (~11%).

2.3.2 Pseudomonas contaminated (PC) cheeses (Trial 1)

Figure 9 shows the estimated marginal means derived from the average abundance of the 15 dominant genera present in the cheese rind for each sampling day for the Pseudomonas contaminated (PC) samples when compared to the control cheese samples plotted against each sampling day. Unlike the LC cheeses this trial did not reach the 60 day
aging point due to the increased *Pseudomonas* presence on the cheese surface. PC cheeses were only followed for the first 28 days of the ripening period. The only genera that did not have a significant two way interaction was *Alcaligenes* spp. (Figure 8, Panel N), which had no significant differences for either treatment or sampling day. *Yersinia* spp. (Figure 8, Panel A, p=0.056) graphically showed a strong two way interaction for sample day and treatment but statistically was only a trend. This can be due to the number of replications. However the data do suggest that presence of *Pseudomonas* significantly increases the presence of *Yersinia* spp. on days 7 and 28. All other major genera identified in trial 1 had a highly significant interaction for treatment and sampling day. More specifically presence of *Pseudomonas* on the cheese rind from day 0 significantly decreases the presence of *Halomonas* spp., *Lactococcus* spp., *Staphylococcus* spp., *Microbacterium* spp., *Streptococcus* spp., *Facklamia* spp., *Arthrobacter* spp., *Alcaligenes* spp., and *Vibrio* spp. On the other hand it significantly increases the presence of *Leucobacter* spp., *Morganella* spp., and *Vagococcus* spp., In the case of *Providencia* spp. the data shows that in the presence of *Pseudomonas* this genus grows faster when compared to the CC cheeses. These shifts can alter the organoleptic properties of the cheese and also have an effect on the proper development of the microflora.

For the phylum *Firmicutes*, *Pseudomonas* spp. increased its presence on day 14 (18.5%). *Facklamia* spp. showed a continuous increase in presence reaching 8.4% by day 28 in un-inoculated cheeses, but that increase was not seen in inoculated ones. *Vagococcus* spp. average abundance increased to 6.2% by day 14 in the PC cheeses but was reduced to almost 0% presence by day 28. In the UC cheeses, this genus only increased to 2.4% at day
28. *Lactococcus sp.* had an average abundance that was less than 1% presence overall by day 14 in the UC cheeses. In comparison *Lactococcus* average abundance levels reached below 1% by day 21 in the PC cheeses. *Staphylococcus* spp. levels were also reduced by day 14 from 44% to 0.6% for UC cheeses. PC cheeses showed that on day 7 the overall presence of *Staphylococcus* on the surface of the rind was 3.5%. (Figure 10)

Members of the phylum *Proteobacteria*, similar to the LC cheese in trial 2, showed the most dramatic changes. *Yersinia* spp. increased its presence in UC cheeses from 10% to 49% by day 14. At day 28 the presence of this genus was reduced to 24%. In contrast in PC cheeses *Yersinia* average abundance at day 7 was 71%, at day 14 was 31% and by day 28 was 64%. This trajectory was almost identically opposite to the UC cheeses. The most significant change in this phylum was the complete absence of *Halomonas* spp. from the PC cheeses while in the UC cheeses it increased to 51% average abundance by day 28. *Alcaligenes sp.* average abundance increased in PC cheeses at day 21 to 23%. This increase was not shown in the UC samples (Figure 11).

In the phylum *Actinobacteria* the major change was the lack of presence of *Microbacterium* spp. in PC cheeses. In the UC cheeses *Microbacterium* reached 7.7% average abundance at day 21 and 3% at day 28. The only other change that was observed in this group was a slight increase in average abundance of *Leucobacter sp.* (0.7%) at day 28 for the PC cheeses (Figure 12).

### 2.3.3 ITS sequence analysis for PC cheese samples (Trial 1)

Analysis of the ITS sequences for the PC and UC cheeses showed a similar average abundance of microorganisms between the two treatments except on day 21. At that
sampling point PC cheeses showed a lower abundance of *Galactomyces geotrichum* (48% in PC vs. 89% in UC cheeses) and higher abundance of *Dipodascus australiensis* (51% in PC vs. 9.4% in UC cheeses). At day 28 *G. geotrichum* abundance increased to 76% in PC samples and *D. australiensis* decreased to 19%. In contrast the abundance of *G. geotrichum* and *D. australiensis* on UC cheeses at the same sampling day were 88% and 8.5% respectively (Figure 13).

2.3.4 ITS sequence analysis for LC cheese samples (Trial 2)

Fungal presence in the LC cheeses was similar for both LC and UC cheeses. *G. geotrichum* was the predominant species for both treatments ranging from 79% to 90% for UC cheeses and 84% to 89% for LC cheeses. *D. australiensis* was the second most predominant species and regardless of treatment it ranged between 8 and 10% average abundance except on day 63 were *D. australiensis* average abundance in UC cheese samples increased to 17%. *Debaryomyces* genus showed some variation, however at day 63 the average abundance for both LC and UC cheese samples was 3-3.5%. In general this genus was present throughout the aging period for the LC cheeses (ranging from 1.1-4.4%) while in the UC cheese it was found mostly below 1% except on days 7, 14, and 63 (Figure 14).

2.3.5 Comparison between UC and CC cheese samples.

Rind samples were collected on days 16, 31, 43, 52, and 57 post-production from the cheesemaker’s caves from cheeses that were produced from the same batch as the experimental cheeses (Figure 15). At day 14 CC cheeses had higher average abundance of *Yersinia* (88.6%) compared to the UC cheeses that had 49.8%. Increased presence of
Yersinia dominated the microflora of the rind for this cheese in the CC samples until day 35 when Microbacterium abundance levels reached 24%. At day 43 CC cheese samples had an average abundance of 11.2% for Yersinia spp., 59.4% for Halomonas spp. and 15.9% for Arthrobacter spp. In contrast on day 42 UC cheeses had an average abundance of 17.5% for Yersinia spp., 25.5% for Halomonas spp. and 17.1% for Facklamia spp. Towards the end of the ripening process, day 57, the three most abundant genera for CC cheeses were Yersinia (81.3%), Facklamia (10.5%) and Halomonas (3.5%). For UC cheeses at day 63 the three most abundant genera were Yersinia (27.4%), Halomonas (29%) and Leucobacter (11%).

Analysis of the ITS sequences also provided evidence that the UC cheeses had a similar composition of microorganisms but differed on abundance. G. geotrichum was the most abundant microorganism of the surface of the rind for UC with a range of average abundance of 79% at day 63 to 90% on days 35 and 42. One genus, Scopulariopsis was only found in CC cheeses, which reached its highest abundance on day 43 (23%). D. australiensis and Debraryomyces spp. were consistently present in the rind in CC cheeses and in higher abundance than in the UC cheeses. (Figure 16)

2.3.6 Discussion

Organoleptic properties of cheese are largely dependent on the development of the surface flora (Feurer et al., 2004). Undesirable contaminants have the potential to grow immediately if the balance of the cheese microflora is disturbed (Bockelmann and Hoppe-Seyler, 2001). This study provides information about population dynamics of the microbial communities on the surface of a soft washed ripened cheese rind when a contaminant, in
our case *Pseudomonas* sp., or a pathogen, like *L. monocytogenes* is present. Despite the fact that cheeses for both trials were ripened in different aging locations, lab modified chambers vs cheesemakers cave, we were able to produce a product that had a similar composition of microorganisms. Changes in abundance of these microorganisms could be a direct result on the ripening method used between the locations. In the lab emphasis was given to minimizing cross contamination by using sterile, one-use surgical brushes and decontamination of the modified chambers.

The *Pseudomonas* trial (PC) was different from the *Listeria* trial (LC) in the sense that a different wash was used by the cheese maker. Cheese samples that were contaminated with *Pseudomonas* sp. where initially brined by the cheesemaker using a brine that contained a local beer as part of the wash which produced a different profile of cheese. The target for that change was to give the cheese some of the characteristics of the beer. However the samples in the lab started changing color to yellow and were extremely wet and odorous. Further investigation, by the cheesemaker, confirmed that *Pseudomonas* was introduced to the cheeses from the beer which was also contaminated with this microorganism. To combat this organism’s growth we lowered our moisture content to 85% and cleaned all the caves in the lab with 70% ethanol. Unfortunately this first trial had to be stopped at 28 days due to the fact that the *Pseudomonas* presence was well established on the cheese surface which made the product created in the lab not comparable to the product made by the cheesemaker.

For the *Listeria* trial (LC) the cheesemaker washed the cheeses in the usual wash solution they had used for previous production lots with no addition of beer or any other
additive. Cheese samples were aged in our lab in the same manner as in our first trial. Cheese samples showed presence of growth of R2R which gives the characteristic pink color on the rind which is consistent with the aging process of the cheesemaker for this type of cheese. There was no increased moisture retention and the cheeses at the end resembled those made by the cheesemaker.

Despite being inoculated with *Listeria* spp., presence of this pathogen was not identified through our molecular analysis as being among the top 15 predominant genera. Cultural methods that were used for *Listeria* detection on the same cheeses using selective media showed the continuous presence of *Listeria* in the cheese. Levels of *L. monocytogenes* in LC cheeses reached $10^6$ cfu/ml on day 14 and remained at that level for the duration of the aging process. It is probable that the pathogen was not able to be in the group of dominant genera due to the fact that it was introduced at a rate of 100 cfu/cm$^2$ and it was immediately required to compete with other established cultures, like non-starter or ripening cultures, as well as starter cultures that are usually introduced to the milk at levels of $10^8$ to $10^9$ cfu/g. This result shows the importance of cultural methods in the identification of pathogens as well as the use of molecular methods to identify communities. Similar observations about this limitation of culture independent methods for isolation of *L. monocytogenes* was reported by Bernini et al. (2013) who was not able that detect of *L. monocytogenes*, through PCR amplification of the hly gene, in blue veined cheeses but was able to use the BAM method to confirm presence of the pathogen. As an example, the *Pseudomonas* inoculation was identified to be approximately $10^9$ CFU/ml by cultural methods however in our data sets it only reached 23% average abundance of at day
21. At day 7 when starter cultures were still in high concentrations, the abundance of *Pseudomonas* did not exceed 1% overall abundance. This result signifies the major limitation of the molecular approach to pathogen identification.

ITS analysis identified a population of yeast isolates with low diversity. *D. hansenii* was found among the dominant species of yeasts on the surface of the cheese and its presence on the rind is in agreement with previous research by Bockelmann and Hoppe-Seyler (2001). The most abundant yeast found was *G. geotrichum*, which is also found in most red smear soft cheese (Eppert et al., 1997). In general on smear cheeses growth of yeasts is the first step in rind development. The yeast will utilize lactate and increase the surface pH of the cheese from approximately pH 5 to pH 7 (Eppert et al., 1997). When pH increases above 6, coryneforms, staphylococci and Gram negative bacteria will begin to grow and will eventually cover the whole surface of the cheese (Eppert et al., 1997; Bockelmann and Hoppe-Seyler, 2001).

Bacterial diversity based on the 16S rRNA analysis was more diverse than that of yeast populations. *Arthrobacter, Microbacterium* and *Staphylococcus* were present in the rind of our experimental cheeses. These genera have been previously isolated from other smear cheeses (Bockelmann and Hoppe-Seyler, 2001; Eppert et al., 1997, Carnio et al., 1999). *Arthrobacter* has been shown to produce a red-brown color when grown in combination with *Brevibacterium linens* (Bockelmann and Hoppe-Seyler, 2001). In addition, *Arthrobacter* and *Microbacterium* have been shown to have anti-*Listeria* properties (Carnio et al., 1999).
Quigley and others (2012) found *Vibrio* spp. only in soft cheese varieties, which is in agreement with our data. *Vibrio* was one of the genera found on the surface of both our UC cheeses (0.1% average abundance) and LC cheeses (2.3% average abundance). Quigley also found that *Halomonas* was present only on semihard cheeses. In our study *Halomonas* was the second most prevalent genera found, with 27% average abundance on the UC cheeses and 20% average abundance on the LC cheeses. In general members of the phyla *Firmicutes* and *Proteobacteria* were the predominant microorganisms found in washed rind cheeses. Our results agree with the previous findings of Wolfe et al., (2014) and Lusk et al., (2012), where it was shown that these phyla are found predominantly on high moisture cheeses and in raw milk cheeses (Delbés et al., 2007).

Wolfe and others (2014) in their study showed that the best predictor for microbial community composition in cheese rinds is moisture content and not geographical region. These authors found that in washed rind cheeses *Proteobacteria* were in high abundance, which data from our study also seems to suggest. In addition, Wolfe et al. (2014) showed that there are widespread interactions between bacteria and fungi during cheese ripening especially with members of the genera *Halomonas, Pseudomonas* and *Vibrio* which were found on our cheese rind, but also with *Pseudoalteromonas* and *Corynebacterium*. The differences observed in the composition of microorganisms in our study could be a direct result of the aging facility. Although we were able to reproduce the aging conditions that the cheesemaker uses for this cheese, aging of our cheese samples was done in a modified and initially sterile environment. Cheesemakers have the advantage of using an aging room that can be easily regulated and in many cases has been colonized with the indigenous flora.
from previous batches of cheese. Our results showed that use of different caves did not introduce a bias among different groups of cheese samples used in our experimental trials. Our data also suggest that ripening the cheeses in different incubators does not produce a bias based on the incubator. It does not, however, indicate that caves and incubators produced the same microbial communities.

The presence of *Lactococcus* bacteria in our LC and UC cheeses showed that it survived on the rind for approximately 14 days after which the presence of these genera on the rind ranged 6 -0.1% average abundance. Previous research has extensively focused on these microorganisms for their ability to produce lactic acid and many strains of *L. lactis* have been evaluated for their anti-*Listeria* properties (Cogan et al., 1997; Sulzer et al., 1991; Abbe et al., 1994; Saubusse et al., 2007; Millet et al., 2006). Although the *Lactococcus* cultures used in our experiments were marketed as protective cultures against *L. monocytogenes*, it is important to state that very few such cultures are currently commercially marketed, which highlights the difficulty in producing such cultures. Data show that the hurdle effect on complex microbiota yields variable results against the inhibition of *L. monocytogenes* (Irlinger and Mounier, 2009).

When comparing mainly spoilage organisms like *Pseudomonas* to a human pathogen like *Listeria* we can see that these microorganisms can have completely different effects on the surface of washed rind cheeses. In the case of *Pseudomonas sp.* the cheese did change appearance and was easily identified as being contaminated. On the other hand that did not happen with *Listeria sp.*, which was able to survive during the aging period.
This can be a direct effect from the extracellular activity of lipases and proteases that *Pseudomonas sp.* can produce (Arslan et al., 2011).

New advances in the identification of bacteria and fungi through use of high-throughput sequencing, has revolutionized the field of microbial ecology. With these methods, researchers can efficiently investigate microbial communities from a variety of samples (e.g. soil, gut, dairy and other food environments) which provides a unique insight into the microbial diversity and dynamics (Quigley et al., 2012). Microbial communities found on cheese rinds are extremely difficult to study due to the high species diversity, low culturability, and inability to stimulate their natural environment (Wolfe et al., 2014). Our main conclusions from this study are that molecular methods might not be the ideal method for initial identification of pathogenic microorganisms in cheeses due to the high abundance of indigenous microorganisms. Instead they should be coupled with current cultural methods. Our data do beg the question, however, that although *Listeria* can be detected when a cheese sample is incubated in and plated onto a highly selective media, is the organism of public health significance when present in a diverse microbial community such as a cheese rind? Further research will be necessary in order to fully explore this question.
<table>
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<th>Strain ID</th>
<th>Source</th>
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<th>Serotype</th>
<th>Lineage</th>
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<td>I</td>
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<td>Food contact surface</td>
<td>DUP-1030-B</td>
<td>1/2a</td>
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Table 2: Overall average abundance of genera found on the surface of washed rind cheeses during an aging period of 63 days in uninoculated cheeses versus those inoculated with *Listeria monocytogenes*.

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<th>Phylum</th>
<th>Genera</th>
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<th>Overall presence in inoculated cheeses</th>
<th>Std Error non-inoculated cheeses</th>
<th>Std Error inoculated cheeses</th>
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<td>1.9</td>
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<td>0.94</td>
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**Figure 1**: Methods used for the trials for soft washed rind cheeses contaminated with *Pseudomonas* (Trial 1) and inoculated with 100 cfu/cm² *L. monocytogenes* (Trial 2).
Figure 2: Microbial communities clustered using PCoA by cheese cave for trial 2 (*L. monocytogenes*). The red dots represent cave 1, yellow dots represent cave 2 and green dots represent cave 3. High overlap between trials reduces bias of the samples due to difference in the cheese caves.
Figure 3: Microbial communities clustered using PCoA by trial. The green dots represent Trial 1 (*Pseudomonas*) samples, blue dots represent trial 2 (*L. monocytogenes*) samples. The trials based on this analysis are separated and clustered within each trial.
Figure 4: Estimated mean values for average abundance of major genera found in washed rind cheese inoculated with a cocktail of Listeria monocytogenes (100 cfu/ml), green line, and uninoculated control cheeses, blue line. A: Yersinia spp., B: Halomonas spp., C: Lactococcus spp., D: Staphylococcus spp., E: Microbacterium spp., F: Leucobacter spp.,
G: *Pseudomonas* spp., H: *Streptococcus* spp., I: *Morganella* spp., J: *Facklamia* spp., K: *Arthrobacter* spp., L: *Vagococcus* spp., M: *Providencia* spp., N: *Alcaligenes* spp., O: *Vibrio* spp., P: taxa with >1% abundance. Significant two way interaction for day and inoculum was found for *Lactococcus* sp. (Figure C, p=0.17) and *Facklamia* spp. (Figure J, p=0.002). Cheese rind samples were taken at 7, 14, 21, 28, 35, 42, 49, 56, and 63 days post inoculation of cheese surface with *L. monocytogenes*.

![70 Day Growth Profile of *Listeria Monocytogenes* on the Surface of a Soft-Ripened, Raw Milk Cheese](image)

**Figure 5:** Surface growth of *L. monocytogenes* on washed rind cheeses during the aging process.
Figure 6: Percentage of organisms belonging to the phylum *Firmicutes* over the aging period of the cheese. Organisms with 0 after the name denote uninoculated cheeses, 100 denotes inoculated cheeses with 100 cfu/cm² of *L. monocytogenes*.
Figure 7: Percentage of microorganisms belonging to the phylum *Proteobacteria* on the surface of the rind of a washed rind cheese. 0 denotes uninoculated cheeses, 100 denotes inoculated cheeses with 100 cfu/cm² of *L. monocytogenes*.
Figure 8: Average abundance for microorganisms belonging to the phylum *Actionobacteria* over the aging of the cheese. 0 denotes uninoculated cheeses and 100 denotes inoculated cheeses with 100 cfu/cm² of *L. monocytogenes*. 
Figure 9: Estimated mean values of major genera found in washed cheese rind inoculated with *Pseudomonas* spp. through the initial brine, blue line, and control cheeses, green line. 

**Figure 10**: Presence of major genera belonging to the phylum *Firmicutes* between cheeses that were inoculated with *Pseudomonas* spp. and uninoculated cheese samples. C: denotes uninoculated samples.
Figure 11: Presence of genera belonging to the phylum *Proteobacteria* found on the surface of the rind of washed rind cheeses that were inoculated and uninoculated with *Pseudomonas* spp. C: denotes uninoculated cheeses.
Figure 12: Presence of genera belonging to the phylum *Actinobacteria* on the surface of washed rind cheeses. Samples were either inoculated or uninoculated with *Pseudomonas* spp. C: denotes uninoculated samples.
Figure 13: Comparison between *Pseudomonas* inoculated and uninoculated cheese rind samples. A: Inoculated cheese samples, B: uninoculated cheese samples.
Figure 14: Fungal presence on the surface of washed rind cheeses based on ITS amplification on both uninoculated (0 cfu/cm$^2$) and inoculated (100 cfu/cm$^2$) during the aging period.
Figure 15: Percent average abundance of bacterial genera in soft washed rind samples for uninoculated lab ripened cheeses (UC) and commercially ripened cheeses (CC) in the cheesemaker's cave. The number next to the cheese sample in the x-axis indicates the...
Figure 16: Percent average abundance of fungal microorganisms found on the surface of soft rind cheeses for uninoculated samples (UC) and cheesemakers samples (CC) on 5 different sampling dates.
CHAPTER 3: FARM SOURCES OF *LISTERIA MONOCYTOGENES* AND IMPACT ON THE MICROBIAL QUALITY OF MILK DESTINED FOR ARTISAN CHEESE MANUFACTURE.
3.1 Introduction

*Listeria* species are naturally found in the environment (Beumer and Hazeleger, 2003). The species is found not only in sewage and fecal matter, but also in decaying plant material. The ability of *L. monocytogenes* to survive under stressful environmental conditions including high salt, low pH and cold temperatures make this pathogen not only very difficult to control, but also extremely persistent in the environment. *Listeria* spp. have been found in both the farm environment as well as in food processing plants and ready to eat foods (Ryu et al., 2013; Carpentier et al., 2011; Nightingale et al., 2004). Due to its high presence and diversity on bovine farms (Nightingale et al., 2004), combined with the artisan/farmstead cheese model of production where small scale farmers produce both raw materials (milk) and final product (cheese) on site, this pathogen is extremely important to control. Risk reduction efforts should be placed on the identification of reservoirs of pathogens such as *Listeria* in the system and the development of practices that reduce the spread of pathogens and, as a result, minimize the risk of contaminating the food supply chain.

Members of the genus *Listeria* are traditionally divided into hemolytic (*L. monocytogenes, L. ivanovii, and L. seeligeri*) and non-hemolytic species (*L. innocua* and *L. welshimeri*). *L. grayi* is still considered to be part of the *Listeria* genus, however it was proposed at one time to represent a new genus, *Murraya*, due to the fact that it is very
different from the other species of the genus (Sauders et. al., 2012). However since the initial description of the genus by Pirie in 1940, new species have been added continuously to the genus. Den Bakker et al., (2014) were able to identify five novel *Listeria* species using whole-genome sequencing, bringing the total number of species for this genus to 15. Of all the species identified to date, *L. monocytogenes* remains the only member of this genus that is pathogenic to humans and animals. *L. ivanovii* is the other species that, although rare, has been shown to cause disease in ruminants (Orsi et. al., 2011).

*L. monocytogenes* is characterized as a facultative intracellular pathogen. In humans, the most severe cases of this disease include encephalitis, septicemia, meningitis and spontaneous late term abortions. The majority (99%) of the infections caused by this pathogen are thought to be foodborne (Orsi et al., 2011). The pathogen is ubiquitous in nature and has been found to exist in many diverse environments including soil, water, vegetation, farm environments, food processing environments, sewage and animal feeds (Sauders et al., 2012). *L. monocytogenes* contamination has been found in many ready to eat products including raw milk, pasteurized milk, soft cheeses, and processed meat and poultry which have all caused outbreaks (Nightingale et al., 2004). Listeriosis cases account for less than 1% of the food borne illnesses reported in the U.S. (Pan et al., 2006) with approximately 1591 cases of human listeriosis with 255 deaths occurring per year (Scallan et al., 2011). Outbreaks from this pathogen are usually associated with consumption of fresh foods as well as fully cooked meat products, fish, poultry and dairy (Muhterem-Uyar et al., 2015) with high mortality rates (20-30%) among immunocompromised patients, pregnant women and the elderly (Hain et al., 2006).
The presence of *L. monocytogenes* in food processing facilities is usually due to its saprophytic lifestyle (Ferreiera et al., 2014) combined with a breakdown of the hygiene barriers placed for personnel and equipment (Almeida et al., 2013). In many cases lack of understanding of where the sources of contamination are in a processing facility leads to unintentional post production contamination of a product. *L. monocytogenes* contamination is primarily post processing (Keto-Timonen et al., 2007). Once established, *Listeria* can persist in food processing facilities for many months or years.

Small scale artisan cheese making facilities are directly affected by this pathogen. Production of artisan cheese most commonly occurs at the farm site. Pritchard et al. (1995) found that dairy processing facilities with a contiguous farm had a higher incidence of *Listeria* when compared to the incidence in processing facilities without an adjacent farm. That creates a direct pathway for transfer of pathogens including *L. monocytogenes*, between the farm environment and the processing facility. D’Amico and Donnelly, (2010), investigated the presence of 4 pathogens including *L. monocytogenes* in small scale artisan cheese production facilities. Their research indicated the need for continuous microbiological monitoring of milk (milk filters), cheese, and the production environment to ensure that the final product is safe for consumption. In addition, factors that are found in association with most small scale producers like pasture feeding, seasonal milking, lack of extending milk holding and small herds sizes contribute to an overall higher quality of milk produced. D’Amico et al., (2008b) found a low incidence of pathogens of concern in raw milk specifically destined for artisan cheese manufacture. Previous research has been focused on preventing the growth and eliminating the pathogen during production through
implementation of safety protocols such as HACCP, however identification of likely farm niches where this pathogen can survive can be valuable for small scale producers when they are creating risk reduction protocols and can lead to overall greater farm hygiene, which in turn can lead to a safer product produced.

This study investigated the presence of *L. monocytogenes* on farms producing milk for artisan cheese manufacture, with the overall goal of identification of the areas where this pathogen most likely survives and management practices that accomplish mitigation of sources of contamination. The conclusions from this study can be used by small scale producers on their farms and in milking facilities to generate safety protocols that will add a barrier to control this pathogen.

### 3.2 Methods

#### 3.2.1 Farms

Dairy farms selected for this study were either supplying milk for off-site cheese production, or they were producing cheese on-site themselves. Farms were selected based on their diverse management practices which included a wide range of approaches such as different bedding styles, methods of milking and overall animal husbandry. Farm A had a small herd (>50 head) and utilized foam pads covered with cedar shavings as bedding. The cows were kept in the barn for the winter but were allowed to graze freely on the fields during spring, summer and fall months depending on the weather conditions. In addition, this was one of the farms in our study that used dry feed for its cows as a method to limit the presence of pathogens in the feed. Milking was semi-automatic. Manure was managed
by a gulley system. Cows were allowed to stay in the barn only for the winter months. In
spring summer and fall the cows were allowed to stay in the pasture and only return to the
barn for milking. Prior to milking, an iodine dip was used for mastitis control. Farm B was
the largest farm in our study that utilized a fully automated milking parlor with iodine
dipping of teats prior to milking. The size of that herd was medium to large (>200 head).
The cows on this farm were continuously kept in the barn. Mechanical scrapers were used
for manure management and kiln dried saw dust was used as bedding. Farm C was also
medium sized (>200 head) and comparable to farm B, with the exception that sand was
used as bedding for part of the farm, and first cut hay was used for other areas. The cows
were allowed to be out on pasture during the spring, summer and fall months depending
on the weather. This farm operated a semi-automated milking parlor and employed an
iodine dip for mastitis control. Farm C was similar in their clean up procedure with farm B
in the sense that they used small farm equipment fitted with a scraper to clean the manure
from the barns, simulating the mechanical scraper system from Farm B. Farm D was also
a medium sized farm with more than 200 head of cows. This farm was the only one of the
4 tested that did not use an iodine dip as a disinfecting step before milking. Instead, they
use the dry method where they clean the cow of visible dirt before milking by the use of
dry paper towels. The bedding used by this farm was dry hay and it was changed regularly.
Farm D also used a gulley system to manage manure. Farm E was a small size farm with a
herd of <30 cows. Apart from being the only other farm in our study to not feed silage this
farm was able to keep their cows longer in the pasture and as a result manure management
was achieved by scraping only. There was not mechanical scraper or gulley system present.
The milking of the cows was semiautomatic and they only employed a dry wipe to remove dirt before milking.

3.2.2 Preparation and sample collection.

Environmental samples for microbiological analysis were collected from farm sites using 3M sterile sponges (3M™ Microbiology, Saint Paul, Minnesota). 10 ml of neutralizing solution (Difco™ D/E Neutrilizing broth, BD, Sparks, MD) was added to the bag 12h prior to collection to rehydrate the sponge and to provide a medium for sample collection. Bags were stored at room temperature prior to use in a disinfected cooler. Ice packs were added to the coolers for sample storage and transportation to and from the farm in order to maintain the temperature of the samples between 5-10°C.

Farm environmental collection sites were separated into three categories: barn, environmental, and milk, based on the location of the sample collection. Samples collected from head rails, side rails, barn floor, cleft, side of barrel and teat end, water bowl, fecal matter, and bedding were designated as barn samples. Samples collected from suction cups, quarter or bulk tank milk samples, milk parlor equipment and pipes, and milk filters were designated as milk samples. Lastly, samples collected from feed, bulk room floor, personnel, drains, foot baths, and soil were designated as environmental samples. Each sample that was collected during the visit was stored in a cooler that was previously disinfected and contained cooling blocks that kept the temperature below 10°C during transport from the farm to the lab.
3.2.3 Media and procedures for isolation and identification of *Listeria* sp.

A modified protocol utilized by the FDA BAM for isolation of *Listeria* from dairy products was followed (Hitchins and Jinneman, 2011). Buffered Listeria Enrichment Broth (BLEB) was used as the primary enrichment medium. The broth contains 30.0g of trypticase soy broth (BD, Sparks NJ), 6.0g of yeast extract (BD, Sparks NJ), 1.35g of monopotassium phosphate (Fisher Scientific, Fair Lawn NJ), 9.6g of disodium phosphate anhydrous (Fisher Scientific, Fair Lawn NJ) and 1.11g of pyruvic acid (Fisher Scientific, Fair Lawn NJ) per liter and was autoclaved at 121°C for 15 min on a liquid cycle. Antibiotics for this broth were added after 4h incubation of the sample to aid the resuscitation of possibly injured cells (Beumer and Hazeleger, 2003).

MOPS BLEB broth was used a secondary enrichment step for the recovery of *Listeria* spp. MOPS-BLEB broth contains 30.0g of trypticase soy broth (BD, Sparks NJ), 6.7g of MOPS free acid (Fisher Scientific, Fair Lawn NJ), 10.5g of MOPS sodium salt (Acros Organics, Fair Lawn NJ), and 6.0g of yeast extract (BD, Sparks NJ) per liter and was autoclaved at 121°C for 15min on a liquid cycle. After it was allowed to reach room temperature 3mL of acriflavin (Sigma, St Louis MO), 5mL of cycloheximide (Fisher Scientific, Fair Lawn NJ) and 8mL of nalidixic acid (MP Biomedicals, Santa Ana CA) were added to the media per liter prior to addition of the sample. One hundred milliliters of the MOPS-BLEB broth was plated onto CHROMagar™ Listeria (DRG International, Springfield NJ) plates in duplicate and they were incubated at 37C for 24h.

Preparation of the antibiotics for the *Listeria* enrichment broths were as follows. Acriflavin and nalidixic acid stock solutions were 0.5% (w/v) and they were diluted in
distilled water. Cycloheximide was diluted in 40% ethanol/distilled water at a final concentration of 1% (w/v). All the antibiotics were filter sterilized using a .45µm nylon syringe filter (Fisher Scientific, Fair Lawn NJ) and were stored at 4 °C for a maximum period of 6 months.

CHROMagar™ Listeria plates were prepared according to manufacturer instructions. Specifically 51.5 g of base was diluted into 1L of ddH2O and autoclaved for 15min at 121°C on a liquid cycle. A vial containing 40 ml of ddH2O and a stir bar was also autoclaved at the same time. The base solution was placed in a water bath set at 50°C. The vial containing the 40 ml of ddH2O was allowed to reach room temperature and 9g of the supplement powder was added. The mixture was agitated for 15 min until the powder was fully dissolved in the water. The supplement mixture was then aseptically added to the base mixture and the agar mixture was dispensed aseptically into petri dishes. Plates were stored at 4°C if not used within 24h.

Samples that were collected using the 3M™ sponges were processed using a modified BAM method. Specifically, 4ml from the collected sponge sample diluent were aseptically aspirated and were used to inoculate a vial containing 36ml of BLEB broth. The samples were then incubated for 4 h at 32°C. After this initial incubation, three antibiotics were added to the broth: cycloheximide (5ml/L), acriflavin (2ml/L) and nalidixic acid (8ml/L). The samples were returned to the incubator for a further 18 h. One hundred µL of this broth was then added to 40mL of MOPS-BLEB broth that already contained acriflavin (3ml/L), nalidixic acid (8ml/L) and cycloheximide (5ml/L). The samples were then placed in an incubator for 18-24h at 35°C. Samples that were not collected with the 3M™ sponge
were subjected to the same analysis with the difference that 25g of the sample was used in
225mL of BLEB enrichment broth.

Following enrichment, 100 µL was streaked to CHROMagar™ Listeria plates, which were incubated for a maximum of 48h at 37°C with each sample plated in duplicate. CHROMagar™ (Beumer and Hazeleger, 2003) is a chromogenic media. *Listeria* β-glucosidase activity, produced by all *Listeria* strains, cleaves the chromogenic substrate in the media producing blue/green colonies. Lecithin present in the media is hydrolyzed by phosphatidylinositol phospholipase C (PIPL-C), synthesized only by *L. monocytogenes* and *L. ivanovii*, which create an opaque halo around the hemolytic species (Jadhav et al., 2012, Jeyaletchumi et al., 2010). Following incubation, plates were examined for presence of blue colonies with or without a zone of clearing. The presence of the clearing zone suggested that the colony was *L. monocytogenes*. Any colony that was blue without a halo present was selected as a possible *Listeria* spp. Approximately 4 colonies of potential *Listeria* spp. per sample where selected based on morphology and streaked again on CHROMagar™ Listeria plates. Following incubation at 37°C for 24h, plates were examined for morphological differences like discoloration or presence of a green halo. Colonies with those morphological differences were not selected for further identification and were classified as other species. Tryptase soy broth supplemented with yeast extract (TSBYE) was used a growth medium for the isolated *Listeria* colonies. It contains 30g of trypticase soy broth and 6g of yeast extract per litter. The broth was autoclaved at 121°C for 15 min and it was distributed aseptically into previously sterilized culture tubes (9ml/culture tube). Isolates were then placed in TSBYE broth and were incubated overnight
at 37°C. Microorganisms grown on this broth were used for either carbohydrate fermentation or for multiplex-PCR identification protocols.

### 3.2.4 Carbohydrate fermentation procedure

Carbohydrate fermentation patterns were determined using methods as described by McLauchlin (1997). The three carbohydrates used were rhamnose, mannitol, and xylose. Each isolate was grown in TSBYE broth for 24 h and 10ul of that inoculum was used to inoculate 3 culture tubes with each tube containing only one type of the carbohydrates mentioned previously. Each liter of the carbohydrate fermentation broth base contained 10.0g of tryptone (BD, Sparks NJ), 5.0g of NaCl (Fisher Scientific, Fair Lawn NJ) and 0.02g of bromcresol purple (Acros Organics, Fair Lawn NJ). Rhamnose (5g/L) and mannitol (5g/L) were added prior to autoclaving while xylose was added after sterilizing the base solution at 121°C for 15min. Xylose (5g/L) was first dissolved in 100 ml of ddH₂O and the mixture was filter sterilized using a .45μm nylon syringe filter (Fisher Scientific, Fair Lawn NJ) into 900ml of base solution at room temperature. Each of the carbohydrate solutions was aliquoted to previously sterilized culture tubes at 10ml per tube and was stored at 4°C for a maximum period of 6 months if not used immediately. Carbohydrate fermentation tubes were inoculated with 10μl of the inoculated TSBYE broth and were placed in the incubator for 24h at 35°C. After the incubation time the carbohydrate fermentation tubes were scored in triplicate.

### 3.2.5 Multiplex PCR method for identification of *Listeria sp.*

A multiplex PCR method was adapted from the method developed by Ryu et al., (2013). Primers, target genes, and primer concentrations for a 50μL reaction are shown in
Table 1. One milliliter of the TSBYE broth, after incubation, was aliquoted to a 1.5 ml microcentrifuge tube (USA Scientific, Ocala, FL). The tubes were centrifuged for 10 min at 4000 x g using a Minispin fixed angle centrifuge (Eppendorf, Hauppauge, NY). Media was aspirated and discarded and the pellet was resuspended in 100µL TE buffer (USB Corp. Cleveland OH). The tubes were vortexed at high speed until the pellet was completely resuspended. DNA was extracted using the E.Z.N.A.Bacterial DNA Kit (Omega Bio-Tech, Norcross, GA) according to the manufacturer’s centrifuge protocol for extraction. DNA was measured using a NanoDrop 3300 (Fisher Scientific, Fair Lawn NJ) fluorospectrometer. A total of 50.8µL PCR mixture consisted of 4.8µL primers mix, consisting of all primers at different concentrations, approximately 25ng of template and 45µL of PCR supermix (Invitrogen, Carlsbad CA). Multiplex PCR was performed with initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30s, annealing at 60°C for 30s, extension at 72°C for 30s and a final extension at 72°C for 5 min using a PTC-100 thermal cycler (MJ Research Inc., Watertown MA). PCR amplicons were electrophoresed on a 2% precast E-gel® with ethidium bromide (Invitrogen, Carlsbad, CA) for 30 min. A 100-bp DNA ladder (New England Biolabs, Ipswich, MA) was used with each gel run. Gels were visualized using a Versa Doc 4000 MP (BIO-RAD, Hercules, CA) gel imaging system.

3.2.6 Ribotyping

All isolates that were confirmed as *L. monocytogenes* were ribotyped though the use of the DuPont RiboPrinter® System (DuPont-Qualicon, Wilmington, DE), a fully automated system, for further identification and confirmation. Strains that were selected
for this method were grown in TSAYE plates overnight at 37˚C from a single colony to create a bacterial lawn. Initial genomic DNA digestion from restriction enzyme *Eco*RI, generating as many as 500 small fragments. Southern blot is then utilized to detect digests that can hybridize with specific genes targeting the genes that code for ribosomal RNA (Jadhav et al., 2012). The pattern that is created was then compared to the available library to identify the serotype and species.

### 3.3 Results

A total of 266 samples (160 collected from barn sites, 67 from collected from milk samples and 39 collected from environmental samples) were obtained. Eighty eight (33.0%) samples were found to be positive for *Listeria* spp. from all samples and farms tested, with some samples testing positive for multiple species. Overall 16 samples were positive for *L. monocytogenes* (6.0%), 57 samples were positive for *L. innocua* (21.4%), 7 samples were positive for *L. grayi* (2.6%), 6 samples were positive for *L. welshimeri* (2.2%), and 2 samples were positive for *L. murrayi* (0.75%). No samples were positive for *L. seeligeri* or *L. ivanovii* (Figure 1).

Of the 88 samples that tested positive for *Listeria* spp. 61 were from barn samples, 17 from environmental samples and 10 from milk samples (Table 2, Figure 2). Further identification to the species level showed that from the 61 positive barn samples, *L. monocytogenes* was isolated from 10 samples, *L. innocua* was isolated from 38 samples, *L. grayi* and *L. welshimeri* were each identified in 6 samples, and one was identified as *L. murrayi* (Table 2, Figure 2). From the 39 environmental samples tested, 17 were found to be positive for *Listeria* spp. with five of those samples identified as *L. monocytogenes* and...
12 identified as *L. innocua*. From the milk samples a total of 67 samples were tested and 10 tested positive for *Listeria* spp. Only one of those isolates was identified as *L. monocytogenes*, 7 were identified as *L. innocua*, one was identified as *L. grayi* and one as *L. murrayi* (Table 2, Figure 2). The only bulk milk sample that tested positive for *L. innocua* was from Farm E. The only other farm that had milk samples testing positive for was Farm B where *L. monocytogenes* (DUP 1039C) and *L. innocua* were isolated from in the milk filter but not in any milk samples (Figure 3, Table 3).

Farm B had the highest number of isolates recovered, with 5 samples identified as *L. monocytogenes*, 31 samples testing positive for *L. innocua* and one sample testing positive for *L. murrayi* (Figure 3). No farm in this study had detectable levels of *L. monocytogenes* in the bulk talk or in quarter milk samples. Even though Farm C had an equal number of sites (5) that *L. monocytogenes* was isolated from as Farm B (5 different sites) all isolates were of one serotype, DUP-ID 1061 which is a type III lineage (Table 3).

In contrast, Farm B isolates belong to 3 different ribotypes, DUP-IDs 1054, 1039C and 1030A which are all lineage II isolates (Table 3). Data show that the same ribotype isolated from silage (DUP 1039C) and in the milk filter and was also found in the drinking water for the animals. Farm B also had the least *Listeria* spp. diversity with all of the non-pathogenic samples except one identified as *L. innocua*. The only other *Listeria* species that was identified was *L. murrayi*.

Farm A and C were the two farms with the highest *Listeria* species diversity. Farm A had 3 *L. monocytogenes* isolates including DUP 1042, DUP 1045C, DUP 1039, 9 *L. innocua*, 1 *L. grayi*, 3 *L. welshimeri* and 1 *L. murrayi* (Figure 3). Even though on farm A
isolate DUP-1042 (Table 3), which has been implicated with outbreaks in the past was identified as one of the strains, none of the isolates were found in barn samples. The pathogenic isolates were found in an area of the bulk tank room that was damp and had a high traffic area with a foot bath. Continuous entry into the bulk tank room made the area there continuously wet. In Farm C *L. monocytogenes* was found approximately 1-2 cm below the surface of the sand bedding, an area that was continually wet due to a flaw in the bedding design. *L. monocytogenes* isolates were also found in samples from feed, side rail, head rail and water bowl (Table 3). In order for the sand not to spread in the barn through the movement of the cows, the cow lane beds were made from cement with no drain and any liquid that was spilled on the bedding could not escape. The second barn on this farm was very damp and dark which also provided good conditions for the presence of *Listeria*. In farm C apart from the 5 *L. monocytogenes* isolates which grouped to DUP-1061 there were 5 *L. innocua*, 6 *L. grayi*, and 1 *L. welshimeri* isolates recovered (Figure 3).

Farm D was similar to Farm E did not use an iodine dip prior to milking. In this farm 8 samples were identified as *L. innocua* and 2 samples as *L. welshimeri* (Figure 3). *L. monocytogenes* was found in the water supply and on the outside of the milking equipment. The strains from these sites were identified through ribotype analysis as DUP-1045B, 1044A, and 1062D, all of which belong to lineage II (Figure 3, Table 3). This farm employed a manure management system that was similar to Farm A. Farm E had only 5 samples that tested positive for *L. innocua* and that was the only *Listeria* species present in the farm.
Of the 16 *L. monocytogenes* isolates recovered in this study from all farms, 5 were found in areas related with water, either in the rim of the water bowl rim, in water or sediment. These isolates were identified as ribotypes DUP-ID 1039, 1061, 1045B, 1044 and 1062 (Table 3). Two isolates were found on the floor of the bulk room and one in the bulk room drain. Only one isolate was found in bedding (DUP-ID 1061) as mentioned earlier in Farm C and two were found in feed (DUP-ID 1061). The majority of the isolates (5) identified were of lineage III and all were found in Farm C. Lineage II isolates were the found in all other farms and samples (Table 3).

### 3.4 Discussion

Understanding the areas were *L. monocytogenes* can be found in a farm setting can provide information to farmers and regulatory agencies that can help in the development of pathogen reduction programs. Areas that were previously identified as high risk include, floors, drains, soil, vegetation or silage, water, fecal matter and refrigerators (Carperntier et al., 2011; Oliver et al., 2005; Nightingale et al., 2004; Vilar et al., 2007). The ability of this pathogen to grow in cold temperatures, over a wide range of pH values, and at water activity as low as 0.92 make its control very difficult (Carpenter et al., 2011). In raw milk and in the dairy environment, the sources of *L. monocytogenes* previously identified are mainly from contaminated feed and bedding (Hunt et al., 2012). In our study, the only bedding material that tested positive for the pathogen was sand in Farm C. However, this was mainly due to a design flaw that probably increased the numbers of the bacterium to detectable levels.
The importance of contaminated silage as a pathway for spreading the pathogen to many other areas of the farm including the animal is well documented (Vilar et al., 2007; Sauders et al., 2012; Nightingale et al., 2004). This provides to the pathogen the ability to contaminate the barn and machinery used for transporting and distributing the feed, as well as the opportunity to infect the animal. In the case of the latter, the cow could be shedding *L. monocytogenes* as an asymptomatic carrier (Oliver et al., 2005; Nightingale et al., 2004). Shedding of the pathogen in fecal matter can be regarded not only as a source of contamination but also as a risk factor for untreated manure used as fertilizer in pastures. Spreading of contaminated fecal matter will increase the widespread inoculum of this pathogen that is found to be abundant in nature (Oliver et al., 2005). In our study, silage samples from Farms B and C were positive for the pathogen. In Farm C the same ribotype (DUP-1061) was found in all other areas of the farm including the water bowl. This pattern does suggest that the bacterium uses contaminated feed to maintain itself in the barn system as Nightingale et al. (2004) proposed (Hunt et al., 2012).

Results from our study indicate that water bowls, regardless if they are used by a small number of cows, as in Farm C, or used by the herd, as in Farm B, are a major source of contamination and presence of the pathogen. This finding agrees with the study carried out by den Bakker et al., (2014) where most of the *Listeria* isolates that that group discovered were predominantly from water in both agricultural and natural environments. Even though that study identified novel species and the investigators were not looking for *L. monocytogenes*, it demonstrated the ability of the genus to survive in water. In Farm B the same ribotype that was found in the water (DUP 1039C) was found also in the milk.
filter. This suggests a direct pathway of transmission from water to animal to milk. Assuming that there was no contamination from fecal matter during milking, there are two possible pathways to explain the occurrence of this isolate in both water and raw milk product. If the feed is contaminated, then the animal could become infected and in turn shed *L. monocytogenes* into the milk supply and the water supply. This pathway is supported by the fact that almost all water sources used for drinking by the animals had sediments and/or silage, indicating that cleaning and monitoring of those areas is not done effectively. The pathogen has the ability to amplify while in the bovine host without producing any symptoms to the animal or the milk (Nightingale et al., 2004) and the pathogen exploits that avenue to maintain itself in the system. Alternatively, the water supply could already be contaminated and as a result it saves as the source of infection for both animals and plants. Pan et al., (2006) stated that some *L. monocytogenes* strains can survive in the environment for long periods of time (>10 years).

Farm A and E were the only two farms where water was not contaminated. Farm A used only dry feed and not silage for feed. Also, they used small water bowls that were activated by the cow by pressing a paddle when drinking water was desired. Using this system, the farm was not only limiting the amount of water that was stagnant in the bowl but also the number of cows per water bowl (2cows/bowl). This conclusion does suggest that it is most likely silage that is contaminating the water and not vice versa. The fact that more isolates are found in water in agricultural environments might just be a result of management. Usually those wet areas are found in cold dark places which are ideal conditions for persistence this bacterium.
Another aspect that was brought forward by Nightingale et al., (2004) was that emphasis has to be given to the specific isolate and serotype found on farms. In their study, they were able to show that some of the *L. monocytogenes* strains were rarely observed in clinical cases and others accounted for most cases. Specifically isolate DUP-1038B was found in 8 of the 17 clinical cases while DUP-1045A was mainly found in environmental samples. This finding was also supported by Jeffers et al., (2001) where it was reported that human epidemic clone DUP-1038 was most commonly found in outbreaks and that clones DUP-1042 and DUP-1038 accounted for 41% of sporadic human listeriosis cases. DUP-1038 is a 1/2a, lineage II clone while DUP-1042 is a 4b, lineage I clone. A 2002 dairy survey of 861 bulk tank milk samples from 21 states showed that the overall incidence of this pathogen in milk samples tested was 6.5% (van Kessel et al., 2004). More importantly, this study it concluded that 93% of the isolates found in that survey belonged to serotypes 1/2a, 1/2b, and 4b (Oliver et al., 2005; Nightingale et al., 2005). In our study, we did find a DUP-1042 strain in Farm A. The sample was taken from the top of a drain that was located on the floor of the bulk room. However this isolate was not found in the milk or any other area of the farm. Serotype 1/2a was the most frequent serotype found in our study. Carpentier et al., (2011) stated that 1/2a strains have higher attachment ability in reduced media than 4b strains. This could explain why serotype 1/2a was most prevalent in our study. This leads to the conclusion that different *L. monocytogenes* isolates can be adapted to survive in different niches by establishing high population densities. Identification of the strain that is most prominent in each environment can provide valuable information on the strategies that have to be implemented for control of this pathogen.
In his publication, Fox et al., (2011) showed that this pathogen, in the farm setting, has a relatively high prevalence with 19%, particularly in milking facilities. In bulk milk the incidence of *L. monocytogenes* has been reported to range from 1% to >12% (Oliver et al., 2005). In our study bulk milk samples were never found to be positive for the pathogen, but Farm E had a positive sample from their bulk tank milk for *L. innocua* and Farm B had a quarter sample that was positive for the same species. The presence of *Listeria* in milk is influenced by numerous factors including farm size, number of animals, farm management, geographical location and season (Oliver et al., 2005). *Listeria* spp. are more likely to be isolated from March to June while *E. coli* are more likely to be isolated from May to June (Hutchinson et al., 2005). Although Oliver et al., (2005) investigated *Listeria* presence in food plants and reported a significant association between size of the plant and recovery of *Listeria* spp, this finding can be applied in barn setting also where the size of the barn and the number of cows can influence the presence of this pathogen.

Fox et al., (2009) also showed that there is a correlation between the level of hygiene standards and presence of the pathogen. This conclusion can explain our findings for the first visit on Farm B when the scraper was not operating properly and there was more accumulation of manure and waste in the barn than usual. Another hypothesis proposed by Fox et al., (2011) is that increased hygiene levels increase the number of different of *Listeria* spp. present. Farms A, C, and D had a more diverse distribution of *Listeria* species. This can be due to the level of hygiene that is maintained on those farms, preventing the establishment of the pathogen in the farm, or because the pathogen is outcompeted by *Listeria* species that are better adapted to survive in the environment and
as a result they outcompete the pathogen. In contrast, in Farm B the only species that were identified were *L. monocytogenes* (5), *L. innocua* (31), and *L. murrayi* (1). The effect of other organisms on the survival, presence and inhibition of *L. monocytogenes* has been previously investigated in final products, such as use of nisin producing lactic acid bacteria (Ennahar and Deschamps, 2000). Recent advances in detection methods through molecular techniques have allowed researchers to look at whole community interactions in both environmental and food samples (Wolfe et al., 2014). Using this approach future research could focus on investigating the interactions between different species or between *Listeria* spp. and their effect on the survival and persistence of *L. monocytogenes*. Imran et al., (2013) alluded to the possibility that the biodiversity of a system could be used as a barrier against this pathogen.

### 3.5 Conclusions

Results from this study continue to support the fact that contaminated silage can introduce the pathogen in a dairy farm setting. From our data and field observations, we identified that sources of drinking water for the animals should be maintained under hygienic conditions. Due to the fact that most isolates were found in areas that are continually wet, use of water should be kept to a minimum. Protocols that immediately dry areas, through the use of radiant heating, or disinfect the areas, should be adhere to and used to control stagnant pools of water in bulk room floors.

More importantly, this study has shown the importance of continuous monitoring of environmental sites for the presence of *L. monocytogenes* in silage. Although
monitoring of either the raw milk or the final product is still essential, most raw milk that is destined for artisan cheese making is of high quality (D’Amico et al., 2008b). Current data suggests that listeriosis outbreaks are more likely to involve dairy products produced from pasteurized milk (Koch et al., 2010) and stress the importance of environmental monitoring in cheese processing facilities. Similar monitoring should be conducted for feed samples (especially if silage is used) and manure destined to be used as a fertilizer in pastures. Such monitoring is consistent with the intent of provisions of the Food Safety Modernization Act.
<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>Primer</th>
<th>Primer Sequence (5'-3')</th>
<th>Primer concentration (µM)</th>
<th>PCR product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Listeria genus</em></td>
<td>Prs</td>
<td>F</td>
<td>GCTGAAGAGATTGCCGAAAGAAG</td>
<td>0.2</td>
<td>370</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R</td>
<td>CAAAAGAAACCTTTGGAATTGCGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>L. grayi</em></td>
<td>Oxidoreductase</td>
<td>F</td>
<td>GCCGATAAAAGGTGTCCGGGTCGCAA</td>
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<td>R</td>
<td>ATTTGCTATGCICCGAGGCTAGG</td>
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<td><em>L. innocua</em></td>
<td>lin0464</td>
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<tr>
<td></td>
<td></td>
<td>R</td>
<td>TCCTACATAGAACCGCATG</td>
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<tr>
<td><em>L. ivanovii</em></td>
<td>namA</td>
<td>F</td>
<td>CGATTCCTATTTCCCTGGAGC</td>
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<td>463</td>
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<td>R</td>
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<td><em>L. monocytogenes</em></td>
<td>lmo1030</td>
<td>F</td>
<td>GCTTGATTCATTGGATTTGTCG</td>
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<td>R</td>
<td>ACCATCCGGATATCTCAAGCCAACT</td>
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<td><em>L. seeligeri</em></td>
<td>lmo0333</td>
<td>F</td>
<td>GTACCTGCTGAGATGACACA</td>
<td>1.36</td>
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<td>R</td>
<td>CTTGCTTCCATATCCGTACAG</td>
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<td></td>
</tr>
<tr>
<td><em>L. welshimeri</em></td>
<td>scrA</td>
<td>F</td>
<td>CGTIGCACAATAGCAAATCTG</td>
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<td>GACATGCCTGCTGAACCTAGA</td>
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Table 1: Primer pairs sequence, target gene, and expected product size for multiplex PCR used for this study developed by Ryu et al., (2013)
Figure 1: Cumulative presence of *Listeria* spp. in all farms. (Sample size n=266, total *Listeria* spp. positive samples=88 (33%)).
Figure 2: *Listeria* spp. Presence separated by sample collection site. Total number of samples collected 266. Number in parentheses indicates total number of samples per sampling site collected.
Figure 3: *Listeria spp.* per farm samples
Table 2: Distribution of Listeria species by environment sampled for all farms in this study (A-E). Number in parenthesis indicates the total number of samples tested for that environment.

<table>
<thead>
<tr>
<th>Species</th>
<th>Barn (160)</th>
<th>Environmental (39)</th>
<th>Milk (67)</th>
<th>Total</th>
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<td>17</td>
<td>10</td>
<td>88</td>
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<tr>
<td><em>L. monocytogenes</em></td>
<td>10</td>
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<td>16</td>
</tr>
<tr>
<td><em>L. innocua</em></td>
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<td>12</td>
<td>7</td>
<td>57</td>
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<tr>
<td><em>L. grayi</em></td>
<td>6</td>
<td>0</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td><em>L. seeligeri</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>L. welshimeri</em></td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td><em>L. ivanovii</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>L. murrayi</em></td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>2</td>
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Table 3: *Listeria monocytogenes* locations per farm and serotype identification per farm

<table>
<thead>
<tr>
<th>Farm</th>
<th>Number of isolates</th>
<th>Location</th>
<th>DUP-ID</th>
<th>Lineage</th>
<th>Serotype</th>
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<tr>
<td>A</td>
<td>3</td>
<td>Drain in bulk room</td>
<td>1042</td>
<td>I</td>
<td>4b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Personnel footwear</td>
<td>1045C</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Entrance to bulk room</td>
<td>1039</td>
<td>II</td>
<td>1/2a</td>
</tr>
<tr>
<td>B</td>
<td>5</td>
<td>Lane holding area</td>
<td>1054</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Water sediment</td>
<td>1039C</td>
<td>II</td>
<td>1/2a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Milk filter</td>
<td>1039C</td>
<td>II</td>
<td>1/2a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Entrance to bulk room</td>
<td>1030A</td>
<td>II</td>
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<tr>
<td></td>
<td></td>
<td>Silage</td>
<td>1039C</td>
<td>II</td>
<td>1/2a</td>
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<tr>
<td>C</td>
<td>5</td>
<td>Silage</td>
<td>1061</td>
<td>III</td>
<td>4a</td>
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<td>Bedding (Sand)</td>
<td>1061</td>
<td>III</td>
<td>4a</td>
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<td>4a</td>
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<td>4a</td>
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<td>Side rail</td>
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<td>4a</td>
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<td>Water pipe supply</td>
<td>1044A</td>
<td>I</td>
<td>4b</td>
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<td>Water</td>
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<td>N/A</td>
<td>N/A</td>
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CHAPTER 4: FATE OF *ESCHERICHIA COLI* AND *LISTERIA* SPECIES IN DAIRY MANURE AMENDMENTS APPLIED IN SOILS IN THE NORTHEASTERN UNITED STATES.
4.1 Introduction

The Food Safety Modernization Act (FSMA) was signed into law by President Obama on Jan. 11, 2011. FSMA was promulgated in response to multistate outbreaks of foodborne illness, many of which involved leafy greens and other produce (Lynch et al., 2009). The U.S. Centers for Disease Control and Prevention (CDC) identified produce as either the first or second leading vehicle in food-borne disease outbreaks attributed to a single commodity within the United States for the period 2006-2008 (Erikson and Doyle, 2012). Risk rankings of foodborne disease outbreaks associated with fresh produce in the U. S. have identified enterohemorrhagic *Escherichia coli* associated with leafy greens as the leading pathogen-produce vehicle combination, followed by *Salmonella* spp. and tomatoes, and *Salmonella* spp. associated with leafy greens (Anderson et al., 2011). In the U. S., during the period 1998-2008, fresh-cut produce was responsible for 56%, 36%, and 17% of the outbreaks associated with fresh leafy greens, tomatoes, and melons, respectively (Erikson and Doyle, 2012).

Under the FSMA Proposed Rule for Produce Safety, FDA originally proposed standards for use of untreated biological soil amendments, which stipulated a 9-month waiting period between soil application and crop harvest in order to reduce the risk of pathogen contamination on fresh produce. FDA has put this rule on hold in order to generate scientific data to best define a specific interval between manure application and planting of edible crops to ensure food safety. Survival of bacterial pathogens and
concomitant risk of produce are impacted by a multitude of factors including climate, manure source, soil type, geographical location and bacterial populations.

Organic amendments of fertilizers including compost and livestock manures are methods used by farmers in the U.S. as a means to fertilize soils (Jiang et al., 2002). This organic cycling of waste products to agricultural lands is considered a very economical, practical and environmentally beneficial management option. However, improperly fermented, composted or stored manure can create a pathway whereby pathogens can be introduced into the human food chain (Brochier et al., 2012; Santorum et al., 2012; Jiang et al., 2002; Reynnells et al., 2014) and can create major environmental problems by contaminating ground and surface water (Edrington et al., 2009). Pathogens such as *Listeria monocytogenes* have been routinely isolated from sewage, decomposing plant material, and manure (Nightingale et al., 2004). *Salmonella* spp. and *E. coli* have been detected in sewage and in animal manures. Proper composting of animal wastes has been shown to reduce pathogen levels (Lemunier et al., 2005). Most studies that have investigated the survival of these pathogens in manure amended soils have applied large population levels of either pathogens or indicator organisms, which is not representative of the realistic conditions found on farms (Jiang et al., 2002). For instance, Brochier et al. (2012) used more appropriate inoculation levels, applying *Enterococcus* at levels up to 1000 cfu/g, and *C. perfringens* at levels of 100 MPN/g in a long term field experiment that involved compost and manure application to soils, in order to simulate the realistic conditions which exist on farms.
Previous studies have focused on the presence of major pathogenic bacteria such as *L. monocytogenes* and *E. coli* O157:H7 (Hutchinson et al, 2005; Santorum et al., 2012; Jiang et al., 2002). Enteric bacteria are capable of transfer from manure amended soil contaminated with animal waste to edible crops grown of these soils. Several studies have found enteric bacteria on or inside a wide variety of vegetables grown in soils amended with manure or irrigated with waste water. As previously mentioned, FDA initially proposed a 9-month interval (270 days) between time of application of manure and subsequent crop harvesting. In areas like the North Eastern U.S., this timeframe forces the growers in those areas out of their growing season. Later, FDA deferred its decision on this issue until a risk assessment and further research could be completed. The current regulations require a 120 day interval between application of manure and ready to eat crop harvesting, which complies with the USDA National Organic Program Standard for organic farmers (FDA, 2015). The Agency further proposed eliminating the 45 day application interval for properly treated and handled compost (including composted manures), citing that use of compost is safer than raw manure, and that it provides an appropriate level of public health protection. FDA (2013) recommends adoption of scientifically controlled composting processes that can achieve levels of <1,000 MPN fecal coliforms/g of total solids on a dry weight basis.

In the case of *L. monocytogenes*, most cases of human listeriosis involve cross contamination of processed finished products from the food processing plant environment. Infected animals and agricultural environments are rare causes of human infections, but animal-derived food or plant products that are not processed before consumption have been
linked to major human disease outbreaks. The list of products involved includes raw milk, raw meat and raw foods of plant origin. A 1981 outbreak in Nova Scotia that involved 42 human listeriosis cases was linked to the consumption of coleslaw that was harvested from fields that were fertilized with untreated sheep manure from *Listeria*-infected sheep (Schlech et al., 1983; Nightingale 2004). More recently, in 2011, an outbreak of listeriosis was traced to consumption of contaminated cantaloupe. This outbreak involved 126 cases in 28 states that included 30 deaths and 1 miscarriage (Fang et al., 2013; FDA, 2012). This was the first outbreak associated with melon that had five outbreak related subtypes, represented by two serotypes of *L. monocytogenes* (1/2a and 1/2b) (McCollum et al., 2013).

Dairy manure is considered to be the primary source of *E. coli* O157:H7 and reduction in the prevalence of *E. coli* O157:H7 shed in manure is predicted to enhance food and environmental safety. Previous studies on the survival of this pathogen in dairy manure revealed that it is capable of survival up to 42 days at 37°C, up to 56 days at 22°C and up to 70 days at 5°C (Jiang et al., 2002). The results from this study suggest that not only can the pathogen in fact survive in cold temperatures, but emphasizes the importance of waste management. Unfortunately, years of research to understand the epidemiology and ecology of this pathogen in the farm and livestock continuum is still insufficient to provide scientifically based recommendations for pre-harvest intervention strategies (LeJeune and Kauffman, 2005).

Our study had 3 main objectives: (a) To investigate if different soil types have an effect on the survival of generic *E. coli* and persistence of *Listeria* spp. in soils in the Northeastern U.S.; (b) To investigate if dairy manure amended soils affect the survival
levels of these bacteria over time; and (c) to investigate if there is a difference between surface application vs. tilling of manure on the survival of *E. coli* and persistence of *Listeria* spp. over time. *E. coli* and *Enterococcus sp.* are routinely used as surrogates to mimic behavior of pathogens that could be harmful to humans (Entry et al., 2010).

### 4.2 Methods

#### 4.2.1 Cultures

Cultures and methods used in this study were based on those developed by Reynnells et al., (2014). Three strains of generic *E. coli* (TVS 353, 354, 355), originally isolated from Salinas Valley, California, were used in our experiments. These isolates display resistance to rifampicin (80 µg/ml) and in our experiments, were designated *rEc* to differentiate them from generic *E. coli* (*gEc*) naturally present in our soil plots. *rEc* cultures were originally isolated by Tomás-Callejas et al., (2011) and supplied to us by the Environmental Microbial Food Safety Laboratory at the Beltsville Agriculture Research Center in Beltsville, MD 20705. Upon receipt, cultures were maintained at -20°C.

#### 4.2.2 Media

For rifampicin resistant *E. coli* enumeration, MacConkey agar (Neogen Corporation, Lansing MI) supplemented with 8ug/L of rifampicin (MACR) (Sigma-Aldrich, St. Louis, MO) was used. To prepare agar plates, 30g of MacConkey powder (Neogen Corporation, Lansing, MI) was dissolved in 1L of distilled water and autoclaved at 121°C for 15m. The media was then placed in a water bath at 50°C where it was left to
cool. One ml of rifampicin stock solution (80mg) was added to the media before pouring the plates. Media that was not used within 24h was stored at 4°C.

Native and non-rifampicin resistant *E. coli* (*gEc*) were plated onto two different media. The first was a MacConkey agar without added rifampicin (MAC). The second media was CHROMagar ECC (DRG International, New Jersey). This medium can differentiate between *E. coli* and other coliforms due to the presence of chromogenic and fluorogenic substrates for the enzymes β-galactosidase (LAC) and β-glucuronidase (GUD). *E. coli* appear blue (LAC+ GUD+) while other coliforms appear mauve (LAC+ GUD-).

The media was made by dissolving 37g of the powder into 1 L of sterile deionized water. The mixture was brought to a boil with gentle agitation and then was placed in a water bath at 50°C and poured into sterile petri dishes. The plates were left at room temperature for 24h and then they were either used immediately or stored for a maximum of 2 months at 4°C.

Buffered Peptone water (Neogen Corp., Lansing MI), 1X strength, was used as a dilution medium for the field samples. Twenty grams of the peptone powder was dissolved in 1L of distilled water and autoclaved for 121°C for 15 min.

Tryptic soy broth (Neogen Corp., Lansing MI) with rifampicin (TSBR), both single and double strength, was used as the medium for the most probable number (MPN) method. The media was prepared according to the manufacturer’s directions. Briefly, 30g of TSB powder was diluted in 1L of distilled deionized water and autoclaved at 121°C for 15min. For double strength TSB, double the amount of powder was used. The broth was cooled to
room temperature and 1 ml of rifampicin stock solution was added per liter for the single
strength TSB and 2 ml of rifampicin stock solution was added to the double strength TSB.

_E. coli_ medium (Neogen Corp., Lansing MI) was also prepared for the MPN
procedure for enumeration of native and non-rifampicin resistant _E. coli_. The procedure
was identical to the TSB method except that no rifampicin was added to the media. Thirty
seven grams per liter of EC media powder was diluted and autoclaved for 15min at 121°C.
The double strength EC media had 74g/liter of EC powder.

Buffered _Listeria_ Enrichment Broth (BLEB) was used for enrichment of soil
samples for _Listeria_ detection. The broth contains Tryptone soy broth (30.0g), Yeast extract
(6.0g), Monopotassium phosphate (1.35g), Disodium phosphate anhydrous (9.6g) and
pyruvic acid (1.11g) per liter of distilled water, and was autoclaved at 121°C for 15 min.
Antibiotics for this broth were added after 4h incubation of the sample to allow injured or
stressed cells to recover. MOPS-BLEB broth contains Trypticase soy broth (30.0g), MOPS
free acid (6.7g), MOPS Sodium salt (10.5g), and yeast extract (6g/L) per liter and it was
autoclaved at 121°C for 15min. After reaching room temperature acriflavin (3ml/L),
cycloheximide (5ml/L) and nalidixic acid (8ml/L) were added to the media prior to the soil
sample being added. This broth was used a secondary enrichment step for recovery of
_Listeria_ species.

ChromList™ (DRG International, Springfield, NJ) agar was utilized as a selective
growth medium for the isolation and identification of _Listeria_ species. The media was
prepared according to directions from the manufacturer. Briefly, 51.5g of the base was
diluted in 1L of deionized distilled (dd) H₂O and autoclaved at 121°C for 15min. A vial
containing 40 ml of ddH₂O and a stir bar was also autoclaved at the same time. The base solution was placed in a water bath at 50 °C. The vial containing the 40 ml of ddH₂O was allowed to reach room temperature and 9g of the supplement powder was added. The mixture was agitated for 10 to 15 min until the powder was fully dissolved in the water. The supplement mixture was then added to the base mixture and it was dispensed aseptically into petri dishes. Plates were stored at 4°C if not used within 24h.

4.2.3 Antibiotics

Preparation of the antibiotics for the *Listeria* enrichment broths were as follows. Acriflavin and nalidixic acid stock solutions were 0.5% (w/v) and they were diluted in distilled and deionized water. Cycloheximide was diluted in 40% ethanol/distilled water at a final concentration of 1% (w/v). All the antibiotics were filter sterilized using a 0.45µm syringe filter and were stored at 4°C for a maximum of 6 months.

Rifampicin stock solution was made by diluting 8g of rifampicin powder in 100 ml of dimethyl sulfoxide (DMSO) using gentle agitation and slight heat. The solution was then filter sterilized by using a nylon 0.22µm filter. The stock solution (80 µg/ml) was stored at 4C until needed.

4.2.4 Preparation of Dairy Manure extract

Dairy manure was collected from Holstein cows housed at the University of Vermont Paul R. Miller Research and Educational Center (Burlington VT). Manure was autoclaved for 30 minutes at 121 C on a liquid cycle. The manure was diluted 1:10 with dH₂O and filtered to make an extract. Briefly, 100 grams of manure and 900 mL of dH₂O were added to a large (2L) Nalgene bucket. Materials were hand-massaged to break up
clumping and vigorously mixed with a large stir bar for 5 minutes. Two layers of sterile cheesecloth were placed in a funnel. The manure slurry was filtered through the cheesecloth, and solids were hand-squeezed to release as much extract as possible, about ¾ the input H₂O volume. Extract was transferred to a clean 9-L carboy, and an equal volume of dH₂O was added. Carboys containing 3L of diluted extract (1:2) were placed in the autoclave for 1 h at 121°C (liquid cycle).

4.2.5 Preparation of the bacterial inocula

Three strains of generic *E. coli* (TVS 353, 354, 355) were cultured individually in 100 ml TSB supplemented with 80 mg/ml rifampicin (TSBR) at 37°C with agitation. Each 100 ml culture was added to a large carboy containing 3 L of sterile manure extract. Carboys were tightly capped and vigorously shaken. The carboys were then incubated at 37°C for 48 h. Following incubation, carboys were placed at 4°C in a cold room. Populations levels for each strain were determined by plating 100ul of the cultures on MACR with the appropriate dilutions. CFU counts were used to determine the amount of each culture required to use for the “cocktail inocula” composed of all 3 strains used for field application. The carboys were then placed in the cold room until needed (but no longer than 48 hours). To prepare the “cocktail inocula” for field application, appropriate amounts of each *E. coli* strain (depending on their population levels) were added to a backpack sprayer (Solo brand, 4-gallon) to make 13-Liters of inoculum. Sterile H₂O was used as the diluent. Contents of the sprayers were mixed well before an aliquot was removed to determine the *E. coli* population in each sprayer.
4.2.6 Field plots, treatments, application of dairy manure solids and *E.coli* inoculum

Field plots were 2m x 1m, separated by 1.5m (5 ft) alley-ways, in a random complete block (RCB) design with 4 replications per treatment. The treatments were: w/o dairy manure solids (DMS) and w/o *E.coli* inoculum and tilled (T-0-0); surface application of DMS with *E. coli* inoculum application (S-M-E); w/o DMS but with *E.coli* inoculum application and tilled (T-0-E); with DMS but w/o *E. coli* inoculum application and tilled (T-M-0); surface application of DMS with *E. coli* inoculum application (S-M-E); and lastly, tilled plots with DMS and *E. coli* applications (T-M-E).

There were two field sites used to prepare plots, with field sites differing in soil composition. Field site A consisted of Hinesburg B fine sandy loam (sand 60%, silt 10% clay 30%), 3-8% slope. Field site B consisted primarily of Adams B loamy sand (sand 40%, silt 40%, clay 20%), 5-12% slope. The treatments were repeated in each field site. For plots receiving DMS, 2.27kg (5 lbs) aliquots in individual bags were manually distributed onto the surface of each plot before being inoculated with the backpack sprayer at an *E. coli* dose of approximately 1x10⁶ cfu/ml. The inoculum cocktail was dispensed and applied at the rate of 1-L per plot using the backpack sprayer. For the plots that were designated for the DMS to be mixed within the soil, a mechanical tiller was used to mix the manure solids in the soil. The tiller was cleaned using ethanol to avoid cross contamination between treatments.

4.2.7 Sampling procedure from surface and tilled field plots

From plots designated as surface application (not tilled), five subsamples per plot (from random loci along an X pattern on the plot surface) were aseptically collected into
labeled Whirlpak® bags. Samples were taken by inverting a round plastic cylinder, previously disinfected with 70% ethanol, (1cm deep) into the soil surface demarcating the soil sample in the inoculated plot; a single use plastic spoon was used to transfer soil/manure from the demarcated sample area to the bag. Sample collection stakes were placed at the site of collection to prevent re-sampling at the same site. For the plots that were tilled, 3 core samples 15cm in depth were collected randomly and a sample stake was placed on the core imprint so that the area would not be resampled. Samples were transported to the lab and hand-massaged for 30 seconds to thoroughly mix the five subsamples within each bag. Thirty grams from each soil/manure sample bag were suspended in 120ml buffered peptone water (BPW) producing a 1:5 dilution (w/w) that was hand massaged to mix thoroughly.

4.2.8 Enumeration and microbial analysis of samples

Samples T-0-0, and T-M-0 were processed for enumeration of native non-rifampicin resistant *E. coli*. All other samples were processed for enumeration of the rifampicin resistant inoculum. The method used for enumeration of both types of *E. coli* was identical. The difference in methods was the use of rifampicin in the media designated for the *rE. coli* enumeration and the use of EC media broth for the *gE. coli* MPN method instead of TSB. One hundred milliliters of each sample was plated in duplicate at its corresponding plate (MAC/MACR). Plates were incubated at 42°C for 24 h and then individual colonies were counted. If the plates produced too few colonies to count (<20 CFU/plate), the sample was re-plated from the primary culture onto 4 plates using 250ul per plate. Samples were again incubated at 42C for 24h. In the event that the second plating
did not yield an appropriate result the most probable number method (MPN) was utilized on subsequent sampling.

4.2.9 MPN method

Samples that did not yield a minimum of 20 colonies per plate were subjected to the MPN method of analysis. For this method, a 48 well block microplate was used. The blocks have 8 rows with 6 x 5mL wells per row. The first row of wells was filled with 1ml of 2x TSBR or 2X EC (if the samples were being enumerated for the non-rifampicin resistant *E. coli*). To this row, 1ml of the sample per well was added and mixed by pipetting. The subsequent wells were filled with 1.8 ml of either TSBR or EC medium depending on the *E. coli* tested. A serial dilution was performed using 200ul of sample per well. Blocks were sealed with a breathable Easy plate (VWR, Bridgeport, NJ) membrane and incubated at 42°C for 24 h. Each well was then plated onto MACR (*rE.coli*) or CHROMagar ECC (*gE.coli*) plates and incubated for 24h at 42°C. A score out of 8 was calculated for each dilution plated for *rE. coli*, and out of 4 for *gE. coli* and an MPN number was computed using an MPN calculator (MPN calculator provided by Dr. Sharma USDA, Beltsville MD).

4.2.10 Listeria Identification

Presence of *Listeria* in soils and/or DMS was determined using enrichment methods specified by D’Amico and Donnelly (2010). In brief, 4ml of the sample collected were aseptically aspirated and were used to inoculate a vial containing 36ml of BLEB broth. The samples were then incubated for 4 h at 35°C. After this initial incubation three antibiotics were added to the broth: cycloheximide (Sigma) 5ml/L, acriflavin (2ml/L) and nalidixic acid (8ml/L). The samples were returned to the incubator for an additional 18 h. One
hundred µL of this broth was then added to 36 ml of MOPS-BLEB broth that already contained acriflavin (3ml/L), nalidixic acid (8ml/L) and cycloheximide (5ml/L). The samples were then placed in an incubator for 18-24h at 35°C. One hundred µL of the MOPS-BLEB broth was plated in CHROMagar Listeria™ agar. Samples were plated in duplicate and incubated at 37°C for 24H. Confirmation of the Listeria spp. was done using the BAX system (Qualicon Inc., Wilmington, DE) following the protocol from the company for the genus Listeria.

4.2.11 Statistical Analysis

The SPSS statistical analysis program was used to analyze the data. The test used was a repeated measures ANOVA, with day as the repeated measure for the E. coli data. Listeria sp. data were analyzed using the general linear mixed model procedure (logistic regression over time) using the same statistical package.

4.3 Results

4.3.1 Surface plots with and without DMS amendment in both field sites.

In both field sites, the initial inoculum after application on day 0 was measured to be between 3-4 log₁₀ cfu/gdw. Amended plots initial mean value for this site was 3.1 log cfu/gdw, and for unamended plots the mean value was 3.8 log cfu/gdw. Field site A plots amended with DMS supported the growth of the bacterium for days 1 and 3. Bacterial populations in those sites achieved a 2.4 log cfu/gdw increase at day 3. In contrast, the unamended surface plots showed a gradual decrease of the population levels of rE.coli for sampling days 1 and 3. The population levels on those plots on day 3 were 0.6 log₁₀ cfu/gdw.
lower than the levels of the bacterium at day 0. Sampling day 7 mean values for both treated plots were almost identical. DMS surface amended plots had a mean value of 2.9 log cfu/gdw while unamended plots had a mean value of 2.8 log cfu/gdw. A rapid decrease from day 3 to day 7 was seen for DMS amended plots that brought the population levels of \( rE.\ coli \) 0.2 log cfu/gdw lower that the levels at day 0. Unamended plots showed a continued gradual decrease in \( E.\ coli \) populations, and at this date they were, on average, 1 log CFU/gdw lower than their initial concentrations (Figure 1).

Population levels for both surface treatments for site A on days 14, 28 and 56 were very similar. Both treatments had a small decrease in \( E.\ coli \) population levels on day 14 from sampling day 7, with the DMS amended plots mean value decreasing to 2.2 log cfu/gdw, and the unamended plots mean value decreasing to 2.5 log cfu/gdw. Day 14 was the first collection date where the MPN procedure was utilized for all samples due to the fact that direct plating of the samples to MACR with either 100ul or 250ul did not yield countable plates. Day 28 sampling showed a rapid decrease for both treatments with a 3.6 log cfu/gdw decrease \{mean value Day 28=0.2 log MPN/gdw\} for the unamended plots and a 2.67 log cfu/gdw decrease \{mean value Day 28=0.5 log MPN/gdw\}, when compared to day 0. Day 56 sampling results showed a slight further decrease in \( E.\ coli \) populations for both treatment plots. The mean value for the surface DMS amended soils at that sampling day was 0.23 log MPN/gdw (a 2.9 log reduction compared to day 0) and for the unamended plots it was 0.18 log MPN/gdw (a 3.7 log reduction compared to day 0) (Figure 1).
Field site B DMS amended surface plots did not support the same initial increase in *E. coli* populations as observed in site A (Figure 2). Both treatments had initial population levels of *rE.coli* that were similar to those in site A at day 0. Amended plots initial mean value for this site was 3.9 log CFU/gdw, and for unamended plots the mean value was 3.8 log cfu/gdw. At day 1 both DSM amended and unamended plots had a slight increase, 0.1 log cfu/gdw for DMS amended plots and 0.2 log cfu/gdw for unamended plots. For day 3 sampling, the mean value for unamended surface plots at this site was 2.6 log cfu/gdw, which corresponds to a 1.1 log cfu/gdw reduction of *rE.coli* populations. The mean value for amended plots at the same date for site B, 3.9 log cfu/gdw, remained almost identical as for the previous two sampling days. *RE.coli* populations in the unamended plots tested at day seven had a mean value of 3.3 log cfu/gdw while the amended plots mean value remained relatively unchanged.

Population level fluctuations for sampling days 14, 28, and 56 were similar for both treatments at this site. Consistent with Site A, there was a sharp decline in the *rE.coli* population at day 14 and 28. DSM amended plots on day 14 had a mean value of 1.8 log cfu/gdw, a 2 log reduction when compared to the levels of the inoculum at day 0, while unamended plots mean value at the same day was 1.4 log cfu/gdw which corresponds to a 2.4 log reduction when compared to initial inoculum levels. As a comparison, this rapid drop was not observed on site A until day 28. By day 28 on site B both treatments showed a 3.6 log decrease of the *rE.coli* populations and had identical *rE.coli* population mean values which were 0.22 log MPN/gdw for unamended and 0.23 log MPN/gdw for amended
plots. No further changes in population levels at this site for both treatments were observed at day 56 (Figure 2).

Compared to site A, *E. coli* populations from site B for both treatments were not able to be detected through direct plating from day 14 forward. Both sites at day 56 had similar r*E. coli* population levels ranging from 0.18 to 0.23 log MPN/gdw. The sites differed in the initial four days of sampling with site A able to sustain higher populations of the inoculum (approximately 1 log higher) until day 14. Temperature data for these field sites were similar, with both sites experiencing an average of a 5°C decrease from day 0 to day 56 (Figure 4). Therefore, the initial changes could be attributed to the different site characteristics (soil type, slope, etc.).

Sampling did not resume until day 135 when the ground was thawed enough to permit sampling. At that day, there were no positive samples for r*E. coli* that were recovered using the MPN methods. On day 165 samples were processed through the bag enrichment procedure which yielded 4 positive surface samples. Two were from DMS amended plots at site A, with no unamended plots testing positive at this time. In site B there was one sample from each treatment that tested positive.

**4.3.2 Tilled plots with and without DMS amendment in both field sites.**

Tilled plots on site A had an initial decrease in r*E. coli* populations on day 1 (Figure 1). The initial mean value for amended plots for this site was 3.5 log cfu/gdw, and for unamended plots levels were 3.1 log cfu/gdw. At day 1 DMS unamended plots had a significant reduction in r*E. coli* population levels of 1.7 log cfu/gdw, which brought the mean value for these plots to 1.4 log cfu/gdw. In contrast the mean value for DMS amended
tilled plots at day 1 was 2.8 log cfu/gdw, which corresponds to a 0.6 log cfu/gdw decrease. At day 3, unamended plots continued to show decreasing levels of *rE.coli* and had a 1.9 log cfu/gdw decrease when compared to the initial inoculum values at day 0. However day 7 and day 14 sampling revealed a rapid increase of the inoculum populations, which was almost identical to the initial levels for these plots by day 14 (mean value day 14: 3.16 log cfu/gdw).

In comparison, DMS amended plots that were tilled showed a gradual decrease in the population levels of *rE.coli* from sampling day 3 until sampling day 28. At day 28, the mean value for these plots was 1.2 log MPN/gdw, corresponding to a 2.3 log cfu/gdw decrease when compared to initial population levels at day 0. At day 56 both treatment plots showed increases in the population levels when compared to day 28. DMS un-amended plots had the higher increase in *rE.coli* populations. The mean value for those plots was 4.6 log MPN/gdw which corresponds to a 3.3 log increase from day 28. The mean value for these plots at day 56 was even higher than the mean value for the same plots at day 0. DSM amended plots had a 0.6 log cfu/gdw increase (mean value day 56: 1.8 log MPN/gdw) in *rE.coli* population when compared to day 28. Although the MPN procedure was used to enumerate the samples at day 56, the mean value observed for the un-amended plots would suggest that direct plating could have yielded countable plates.

Field site B (Figure 2) tilled DMS amended and un-amended plots did not have an initial decrease as in site A. *R-E.coli* initial population mean values were 3.1 log cfu/gdw for both DMS amended and un-amended plots. The population levels remained unchanged for sampling day 1. By day 7 both treatments showed an increase that was higher than the
initial levels for both treatments. Amended plots showed the highest increase at this date which was a 1.5 log cfu/gdw. At the same date, the un-amended plots had a mean value that was 0.24 log cfu/gdw higher than the initial population level at day 0. From day 7 until day 56, both treatments showed a gradual decline for \textit{rE.coli}. At day 56 the mean population values were 1.1 log MPN/gdw and 0.48 log MPN/gdw for un-amended and DMS amended plots, respectively. The mean values correspond to a 2 log decrease for un-amended and a 2.6 log decrease for amended plots at this site.

In both sites, un-amended tilled plots had significantly higher levels of \textit{rE.coli} as indicated by the mean values at day 56 (Figures 1 & 2). The increase that is seen at field site A at day 56 was not seen at the corresponding plots on site B. Identical to surface plots, sampling was suspended until day 135 because of frozen ground. There was no \textit{rE.coli} found in either site through MPN method on this sampling day. Subsequent sampling on day 165 through bag enrichment showed the same results as day 135 revealing absence of \textit{rEcoli} (results not shown).

4.3.3 Native \textit{E. coli} population levels and \textit{Listeria} sp. presence

Generic \textit{E. coli} population levels and native \textit{Listeria} sp. presence was assessed in un-amended and DMS amended tilled plots with no \textit{rE.coli} inoculum application. Figure 3 shows the population levels for g \textit{E.coli} for both field sites (A&B) from day 0, 14, 28, and 56 using the MPN procedure. The MPN procedure was employed throughout sampling of these plots as low levels of naturally occurring populations were expected. Generic \textit{E. coli} population levels for DMS amended plots had a higher mean value (3.7 log MPN/gdw in
site A, and 2.9 log MPN/gdw site B, Figure 3) than unamended plots (1.2 log MPN/gdw site A, 0.35 log MPN/gdw site B, Figure 3).

Site A amended plots had higher mean gEcoli level throughout the trial than all other plots in both sites. The only time there was a decrease in gE.coli presence was on day 28 were there was a 1.7 log MPN/gdw difference between the mean values from days 14 and 28. Days 0 and 14 had almost identical mean values (3.7 and 3.6 log cfu/gdw respectively). Day 56 g E.coli population levels were almost identical to those at day 28 with a final mean value calculated as 1.9 log MPN/gdw (Figure 3). In comparison, amended plots from site B did not have the same trajectory. Data shows that there was a gradual decrease that almost resembled the profile that was observed for the surface samples of rE.coli. The mean value for these plots was 0.45 log MPN/gdw which represents a 2.4 log decrease when compared to day 0.

Un-amended plots tested for gE.coli did not show similar profiles between day 0 and day 14. Site A plots showed a 1.8 log MPN/gdw increase that was not observed in the corresponding plots at site B. Population levels were almost identical for both sites at days 28 and 56. Mean values at day 56 were 0.44 log MPN/gdw for site A and 0.48 log MPN/gdw for site B. Both these values are similar to the mean value seen for the amended plots at site B. At day 135 the only plots from which recovery of gE.coli was possible was from amended plots from site A.

Listeria sp. presence or absence was tracked on the same plots as the gE. coli on sampling days 7, 14, 28 and 135. Listeria was present in all plots regardless of treatment with DMS (data not shown). Initial investigation of the manure solids that were spread on
the plots showed the presence of *Listeria innocua*. However, since the bacterium was found in plots that were not treated with manure solids it is hypothesized that a native *Listeria sp.* population was established in both fields. None of the *Listeria* isolates that were found was pathogenic. Statistical analysis using the generalized linear mixed model showed no significance between field, DMS amendment or time for *Listeria* survival.

4.3.4 Temperature data.

Figure 4 shows the average weekly temperature for both sites (A&B) at both depths measured. Site A 10cm depth values were higher by an average of 3°C from day 60 until approximately day 120 (ranging from -3.1 to 0.79 °C; 26.4 and 33.4 °F). These days correspond to the months of January and February which was the coldest time period throughout this study. Specifically on day 70 site A at 10cm depth was almost 4°C warmer than all other measurements for both sites. Furthermore at day 112 (week 16) temperature at that depth averaged above 0. Overall there was a 12°C decline in temperature at 2cm (6.8°C on day 7 reaching -5.2°C on day 70) and a 10°C decline at 10cm depth at site A (7.1°C on day 7 reaching -3.1°C on day 70). For site B there was a 13°C decline in temperature at 2cm (6.7°C on day 7 reaching -6.6°C on day 70) and 11°C decline at 10cm depth (5.3°C on day 7 reaching -6.01°C on day 70). Although these values represent the maximum differences, the data suggest that plots at site B and surface temperature at site A had colder soil temperatures present for longer periods of time than the temperature recording for the 10cm depth at site A.
4.3.5 Soil Moisture.

Average percent moisture content was measured using a CR10x with 107 Temperature Probe and 257 Watermark Soil Matric Potential Block (Campbell Scientific, Inc. Logan, Utah). In general moisture content did not have any dramatic change for the first 56 days of the trial. Tilled plots on average retained lower levels of moisture compared to surface plots and DMS amended soils retained more moisture than un-amended soil plots (Figure 5). Tilled plots maintain soil moisture of approximately 15% ±1 regardless of site. The greatest changes were seen between day 0 and day 14 for surface plots. Surface plots that were un-amended had an initial reduction in percent moisture from 11% to 4.3% between day 0 and 7, respectively. In comparison, surface plots that were amended with DMS had an initial increase in moisture content that reached 23% on site A and 20% of site B.

4.3.6 Statistical Analysis

Statistical analysis of the data for the first 56 days of the trial using a repeated measures ANOVA procedure showed that there is was a significant three-way interaction between tillage, DMS and time for site A (F(16)=2.919, p=0.018) and a trend for site B (F(16)=2.214, p=0.056). Statistical significance for site B was found in the two way interaction between tillage and time (F (16) =9.359, p=0.0001). In both sites the data suggest that application of manure on the surface of the soil and not tilling it into the soil results in significant reduction of the population levels of *E.coli* over time. Additional sampling is required to investigate if the results in site B are indeed a trend since the p value obtained is very close to being significant. When all the data were compared,
combined for farm site, a significant three way interaction was seen for farm site, tillage and time (F(32)=4.324, p=0.001). The results of the statistical analysis indicate that there is significant difference between the two test sites. Generic *E.coli* analysis showed a significant 3 way interaction for field site, DMS and time (F(16) =3.944, p=0.016). These results suggest that there is a significant difference in the survival *gE coli* in the soil if the manure amendment is tilled into the soil, but this effect is dependent on type of soil and possibly location.

**4.3.7 Discussion**

The U.S. EPA has established microbiological standards through the Part 503 rule for biosolids, which must contain <1000 MPN/gdw for fecal coliforms (FDA, 2015). Based upon this definition, most of our study plots met this criterion 28 days after manure application. Dairy manure in the form or raw, slurry or composted is routinely applied to land as a crop fertilizer (Jiang et al., 2002). It was estimated in 2005 that 132 million metric tons of dairy manure is produced annually in the United States and it is then applied to approximately 9.2 million hectares of land (Edrington et al., 2009).

This study investigated the importance of soil type on the survival of enteric bacteria (*E. coli*) and *Listeria* spp. Our data suggest that soil type and location can greatly influence the survival of microorganism in soils and is in agreement with previous research that suggests that physical characteristics of the soil affect the microorganism’s survival rate (Lau and Ingham, 2001; Jamieson et al., 2002; Zalenski et al., 2005). Soil moisture retention seems to be the soil property that has the greatest impact on bacterial survival and is directly linked to particle size and distribution of organic matter content. In studies using
E. coli O157:H7 lower death rates were seen in soils with higher matric potential, which is the difference in water potential between a system and its equilibrium dialysate when both are at the same height, temperature and are subjected to the same external pressure (Whalley et al., 2013) Increased fine soil particle presence also has a positive effect on the survival of enteric pathogens in soils (Zaleski et al., 2005) due to the fact that those soils have an increased ability to retain nutrients. (Jamieson et al., 2002). Population levels of \( rE.\ coli \) had lower death rates in sandy loam soils than in loamy sand soil type in tilled plots. The difference between these two types of soils is that the loamy sand soil contains more silt and less sand than the sandy loam. From the previous research on soil types it was expected that site B should support a slower rate of \( E. \ coli \) mortality than site A. The other difference that these field sites had was slope. Site A had a 3-8% slope while site B had a slope of 5-12%. This difference can have an effect on the moisture retention and transport of bacteria in the soil. In addition, site A was surrounded with trees to the north and east while site B had no tree cover. This provided site A with some shade which has been reported to increase survival time of \( E. \ coli \) (Zaleski et al., 2005).

At day 56 in site A, our data showed elevated levels of \( rE.\ coli \) in tilled un-amended plots that was not seen in corresponding plots at site B. The event could be a direct response of the bacteria to increased moisture content, Three days prior to sampling, the sites experienced a rainfall event, although we were unable to measure soil moisture content at that time. Moisture is a determining factor for the survival of \( E. \ coli \) in loamy soils (Jamieson et al., 2002). The rapid increase in the population of \( rE.\ coli \) supports Zaleski et al. (2005) proposed hypothesis for regrowth. This proposed hypothesis states that even
though a pathogen population has decreased, if it is at or above critical threshold, a rainfall event can trigger regrowth.

Plots that had surface application of dairy manure solids had \( rE \) coli populations that were below detectable levels when determined by direct plating on day 14. The results suggest that either there was a run off event that reduced the density of the population, or that \( rE. \) coli were not able to survive the soil temperature on those days. Previous research has shown that increased temperature generally reduces the survival rate of a microorganism (Jamieson et al., 2002). Enteric pathogens such as \( E. \) coli O157:H7 have been shown to be capable of survival up to 70 days at 5°C in dairy manure. Based on those findings, reported by Jiang et al., (2002), and the fact that the sites were experiencing temperatures between 0 and 6 °C, survival of the bacterium was expected to be seen throughout the duration of the study. The gradual but continuous reduction in \( rE. \) coli in the plots on both sites could be a result of the application method. Dairy manure solids were not mixed with the bacterium. The bacterium was sprayed on top of the plots after the solids were added to the plot.

Although temperature might not have played a significant role at the start of the trial, previous research has shown that the freeze-thaw events can reduce bacterial population levels in soils (Jamieson et al., 2002; Zaleski et al., 2005). This can explain the results from days 135 and 165 when recovery of \( rE. \) coli was not possible through bag enrichment. The result is also supported from the data for native \( gE. \) coli. At day 135 the only plots that were found to be positive for presence of native \( E. \) coli were the tilled plots in site A with DMS amendment. The temperature for those plots at a 10cm depth was
calculated to be approximately 3°C higher than in site B. In addition, native \( gE.\text{coli} \) had already established a population in the manure solids and was not introduced in the same manner as \( rE.\text{coli} \). This result emphasizes the importance of manure management and animal husbandry in farms in order to eliminate introduction of pathogens through manure spreading.

The method of application of \( E.\text{coli} \) increased the effect of competition from other resident microorganisms and can exert pressure on introduced bacteria. Enteric bacteria that have been introduced to the soil need to compete with resident bacteria for nutrient and moisture availability (Jamieson et al., 2002; Zaleski et. al., 2005). In this case, the manure was tested prior to application and it was found to contain both \( E.\text{coli} \) and \( Listeria \) spp. as resident microflora. The increase in competition among resident \( gE.\text{coli} \) and \( rE.\text{coli} \) was not seen in the tilled plots probably due to the fact that tilling redistributed the bacteria in the soil and allowed for better infiltration of moisture (Ceja-Navaro et al., 2010).

Manure addition was not seen to be significant in affecting survival of \( rE.\text{coli} \) populations over the duration of this study, however tilling of soils was seen to be highly significant. Results show that the plots that were tilled with no DMS amendment had higher levels of \( rE.\text{coli} \) at day 56. This could be directly associated with the levels of competition among other bacteria in the manure and type of manure used. Concentration of indicator and pathogenic organisms has been shown to differ depending on animal type, waste storage (Jamieson et al., 2002) and animal health (Gagliardi and Karns, 2000).

Recently, a study conducted by Entry et al., (2010) investigated the effect of tillage and dairy manure application on the survival of bacterial pathogen indicators. The study
measured the presence of *E. coli* and *Enterococcus* spp. in soil and fresh radish peel for 84 days post initial application of the manure. Manure was applied at two concentrations, a high and low, and it was tilled to either 20 cm or 10 cm in depth. The authors were able to detect *E. coli* up to 54 days post manure application but on day 84 they were not able to detect any *E. coli* in the soil or the rhizosphere of the plants. The study did find that *Enterococcus* spp. were able to be isolated for the total duration of the study in both soil and rhizosphere. Their data is in agreement with our findings for *rE. coli*. Based on their findings, Entry and others (2010) suggested an interval of 120 days prior to planting as a way to reduce the incidence on pathogenic bacteria in fresh produce, and results from our studies would support this recommendation.

*Listeria* spp. in this study were followed for some of the sampling dates in order to access their presence in the soil and to determine if manure addition has an effect on persistence. Our data suggest that *Listeria* spp. were constantly present in the soil regardless of dairy solid manure addition. Nightingale et al., (2004) showed that the presence of *Listeria* spp. in manure is directly associated with the ingestion of contaminated feed. The bacterium then amplifies in bovine hosts and is subsequently disseminated back into the environment with the practice of manure spreading. An outbreak of listeriosis in Nova Scotia in 1981 (Schlech et al., 1983) was linked to cabbage grown on fields where manure from *Listeria*-infected sheep had been applied.

The data for the microorganisms tested in our study show that *E. coli* cannot be used as indicator species for *Listeria* spp. Assessment of different pathogens and discovery of indicators that behave closer to the pathogens of interest in both the soil environment
and the animal fecal matter is needed. Contrary to our findings, Brochier et al., (2012) concluded that the *Enterococcus* spp. used as indicators in their plots did not show any significant difference in survival when compared between amended and un-amended soils with either compost or manure. This result suggests that in some cases the actual pathogen needs to be investigated in order to understand its ecology in the system due to the fact appropriate indicators might not be available. The same study also found that the presence of pathogens such as *L. monocytogenes* were absent or below detection limits. Brochier et al., (2012) used cultural methods so the detection of the pathogens in the sample depends on the ability of the microorganisms to grow in the lab environment. Molecular methods can be employed in such cases to work in conjunction with cultural methods when issues of low detection arise.

Management of manure waste by farmers is extremely important in order to reduce pathogen presence in the soil, plants and ultimately in the human food chain. Nightingale et al., (2004) showed the importance of using non contaminated feed. Most farms do not manage their manure waste stores as batches. Usually new manure is added constantly on top of the old, which continually replenishes the inoculum of microorganisms. A better approach rather than the interval between planting suggested by Entry et al., (2010) and the FDA is to properly manage the manure wastes prior to field spreading. For example, seasonal differences should be taken into account. *Listeria sp.* are more likely to be isolated from March to June while *E. coli* are more likely to be isolated from May to June (Hutchinson et al., 2005). Nicholson et al., (2003) showed that *Listeria* spp. can survive in slurries for up to 3 months while it can only survive for one month in solid manure heaps.
4.4 Conclusions

The main conclusions of this study are that soil type, location and physical characteristics have a significant role in the survival of bacterial populations of *rE. coli*, *g E. coli* and *Listeria* spp. in soil. Dairy solids application does not seem to have an effect unless the bacterium of interest is found in the manure and is not artificially introduced. Tilling of soils results in increased survival of the bacterial population due to the fact that it increases soil pore size and facilitates moisture entry, which in turn has been shown to increase bacterial survival rates.

A limitation of this study is the fact that *rE. coli* were introduced to the soil through a sprayer and not through the dairy manure solids application. Future studies should consider changing the methods of indicator growth and application in field soils to reflect standard farm procedures. Further investigation is required to evaluate waste management procedures and dairy herd management as these practices have a direct effect on the presence of pathogens in manure destined for fertilizer use.
**Figure 1:** Survival of *E. coli* at location A, measured as log CFU(MPN)/gdw, between tilled and non tilled plots with and without DMS amendment. Temperature was measured for the duration of the study at 2cm and 10 cm depths. The average weekly temperature is plotted on the secondary Y axis for each of the sampling days. DMS: Dairy manure solids, gdw: gram dry weight of soil.
Figure 2: Survival of *E. coli* at location B, measured as log CFU(MPN)/gdw, between tilled and non-tilled plots with and without DMS amendment. Temperature was measured for the duration of the study at 2 cm and 10 cm depths. The average weekly temperature is plotted on the secondary Y axis for each of the sampling days. DMS: Dairy manure solids, gdw: gram dry weight of soil.
Figure 3: Presence of generic *E.coli* (*gE.coli*), measured as log CFU(MPN)/gdw, between the two experimental fields (A and B) and between tilled DMS amended and unamended plots. DMS: Dairy manure solids.
Figure 4: Average weekly temperature change for both experimental fields (A and B) from two different soil depths (2 cm and 10 cm) for the first 165 days of the trial.
Figure 5: Percentage soil moisture for both field sites (A and B) from day 0 through day 56. S: Surface application plots, T: Tilled plots, w DMS: Dairy manure solids amendment, w/o DMS: no addition of dairy manure solids.

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