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An Environmental Approach To Food Safety Assessment Using Artisan Cheese And Fresh Produce As Model Systems

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AN ENVIRONMENTAL APPROACH TO FOOD SAFETY ASSESSMENT USING ARTISAN CHEESE AND FRESH PRODUCE AS MODEL SYSTEMS

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Abstract

This dissertation examined recently issued regulatory standards using cheese and produce as model systems. FDA's 2015 Domestic and Imported Cheese and Cheese Products Compliance Program Guidelines (CPG) *E. coli* standards on cheese safety, and the extent to which these standards affect domestic and imported cheese commerce, was assessed. Results from FDA's Domestic and Imported Cheese Compliance Program for samples collected between January 1, 2004 and December 31, 2006 were analyzed. Of 3,007 cheese samples tested for non-toxigenic *E. coli,* 76% (2,300) of samples exceeded 10/g, FDA's target for regulatory activity. In cheese samples containing *E. coli* levels of 10/g and 100/g, there was no statistically significant association with presence of *Listeria monocytogenes*. However, associations between *Staphylococcus aureus* levels of 10,000 CFU/g and presence of *Salmonella* and *L. monocytogenes* were statistically significant, indicating that EU regulations targeting *S. aureus* as the pathogen of concern may be more appropriate than *E. coli* for cheese safety assessment.

Compost amended soils in the Northeastern U.S. were assessed for the presence and survival of *E. coli* and *Listeria* spp. against FDA Food Safety Modernization Act (FSMA) requirements. Manure and poultry litter-based biological soil amendments of animal origin (BSAAO) must achieve pathogen reduction to reduce risk of pathogen contamination on the harvested produce. Two trials of replicated field plots of loamy (L) or sandy (S) soils were tilled and un-amended (NC) or amended with dairy compost (DC), poultry litter compost (PL), or poultry pellets (PP). Colony count and most probable number (MPN) methods were used to determine persistence of *E. coli* in these plots over 104 days post-inoculation. Detection of indigenous *Listeria* spp. were also examined in all plots. Higher *E. coli* populations were observed in PL and PP amended soils when compared to DC and NC plots. *E. coli* was detected at low levels on radish crops, where PL treatments encouraged greater levels of survival and growth than DC or NC. Study results verify that a 120 day interval following BSAAO application should be sufficient to ensure food safety of edible crops subsequently planted on these soils.

The sensitivity of environmental monitoring methods and collection formats were evaluated to identify optimal procedures for detection of *Listeria* spp. on product contact surfaces within artisan cheese production environments. Four environmental surfaces (dairy brick, stainless steel, plastic, and wood; n=405/surface type) were inoculated with *L. innocua*, *L.m.* ATTC® 19115 and *L.m.* 1042, at high (10^6-10^7/cm^2) and low (0.1-1/cm^2) target concentrations. Inoculated surfaces were swabbed with World Bioproducts© EZ Reach™ and 3M™ environmental swabs (3M™). Five enrichment and enumeration methods were used to compare sensitivity of recovery between environmental swabs. All swab formats performed equally on all environmental surfaces at high target concentrations. At low concentrations, Petrifilm™ and WBEZ swabs recovered *Listeria* spp. from 87.5% of plastic, stainless steel, and dairy brick surfaces, but only 62.7% of wooden surfaces; recovering 14.8%, 77%, and 96.3% of cells from initial inoculations of 0.01, 0.1, and 1/cm^2, respectively . Our data demonstrate that results may be discrepant due to variation in the porosity of environmental surfaces and should be taken into consideration when implementing environmental sampling plans. Results from this thesis can be used to inform regulatory policy and help to achieve improved food safety.

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CHAPTER 1: COMPREHENSIVE LITERATURE REVIEW

Pathogens of Concern

Previous risk assessments, compliance guidelines, regulatory stipulations implemented by FSMA, and completed studies, as well as studies that will be discussed, state that the four primary pathogens of concern in ready to eat cheese and cheese products, include: *Staphylococcus aureus*; Enterotoxigenic *Escherichia coli* (*E. coli*); Shiga-toxin producing *E. coli* (O157:H7); *Salmonella* spp.; and *Listeria monocytogenes.* The FDA also considers *Bacillus cereus* as additional pathogen of concern. In soils, environmental samples, and produce, Shiga-toxin producing *E. coli* (O157:H7), *Salmonella* spp., and *L. monocytogenes* are major pathogens of concern.

Listeria monocytogenes

Genus *Listeria*

Listeria are Gram positive, non-spore forming, catalase and oxidase positive, facultatively anaerobic, short rods (0.5µm x 0.5-2µm), with rounded and sometimes coccoid ends. Cells occur individually or in long filament formations. Peritrichous flagella allow the bacteria to only be motile at 20 to 25°C and not at the optimum growth temperature of 30 to 37°C (FDA, 2012; Low and Donachie, 1997).

The seventeen species of *Listeria*e are *L. welshimeri*, *L. grayi*, *L. innocua*, *L. ivanovii, iL. aquatica, L. booriae, L. cornellensis, L. fleischmannii, L. floridensis, L. grandensis, L. marthii, L. monocytogenes, L. newyorkensis, L. riparia, L. rocourtiae, L. seeligeri,* and *L. weihenstephanensis* (Weller et al., 2015). *L. ivanovii* is known as a non-human pathogen, although isolated cases where *L. ivanovii* caused disease to humans

were documented (Cummins et al. 1994). *L. monocytogenes* affects both humans and animals (Low and Donachie, 1997; Farber and Peterkin, 2018) and was the most prominent pathogen studied for this thesis. *L. monocytogenes* is a bacterial foodborne pathogen that causes listeriosis, an invasive illness that occurs in immunocompromised individuals such as infants, older adults, and pregnant women (Silk et al., 2012). Serotyping is used to differentiate *L. monocytogenes* and recognized serotypes include 1/2a, 1/2 b, 1/2c, 3a, 3b, 3c, 4a, 4ab, 4b, 4c, 4d, 4e (Nadon et al. 2001; Ryser 1999). Strains of *L. monocytogenes* most commonly involved in outbreaks belong to serotype 1/2a, 1/2b, and 4b, with serotype 1/2a and 1/2b strains isolated from some of the largest listeriosis foodborne outbreaks that occurred in the U.S. (Nyarko et al., 2017). *L. monocytogenes* is a hardy psychrotroph that can tolerate temperatures between 0.4 and 45ºC and grow in foods with water activity levels between 0.90 and 0.97, and pH values between 4.3 and 10 (Farber and Peterkin, 2018; Nolan, Chamblin, and Troller, 1992; Ryser, 2001). *L. monocytogenes* is relatively halophilic with the ability to grow and survive in salt concentrations of up to 10%. Cases have been identified where *L. monocytogenes* survived in salt concentrations of 26% for up to four months under refrigeration parameters (Ryser, 2001).

Virulence

Listeriosis is the disease caused by *L. monocytogenes*. Listeriosis was first described by Murray and colleagues in 1926 (Ryser, 1991). Symptoms include meningoencephalitis and abortion in ruminants such as sheep and cattle (Shank et al., 1996). Nyfeldt first discovered human infection caused by listeriosis in 1929, as a zoonitic disease acquired

from infected animals.(Farber and Peterkin, 1991). Listeriosis is characterized by severe clinical symptoms that include meningitis, encephalitis, septicemia, neonatal sepsis, and preterm labor in pregnant women. A non-invasive form of listeriosis can also manifest and often cause febrile gastroenteritis with flu-like symptoms in immunocompromised and healthy populations (Nyarko, 2017) including include the elderly, fetuses, cancer patients, human immunodeficiency virus (HIV), patients, organ transplant recipients, and individuals receiving corticosteroid therapy. The first reported foodborne outbreak occurred between 1949 and 1957 in Germany (Ryser, 1999). *L. monocytogenes* was recognized as an etiological agent of foodborne listeriosis in the 1980's. In 1999, listeriosis accounted for only 0.02% of foodborne illnesses per year and out of those cases 27.6% resulted in fatalities (Mead et al., 1999). Today, approximately 1591 cases of human listeriosis occur in the United States annually, with 255 of these cases ending in death (Scallan et al. 2011, CDC, 2017a) . The probability and risk of foodborne illness related to listeriosis relies heavily on the predisposition of these populations, the food composition, and the strain virulence of individuals (Schlech, 2000); CAC, 2000).Virulence of *L. monocytogenes* is initiated through phagocytosis by host cells, where the bacteria multiplies within the cell cytoplasm and can invade neighboring cells. Five genes associated with virulence are *plcA*, *hly*, *mpl*, *prfA* and *plcB*, which are located in the primary pathogenicity island *prfA*-virulence gene cluster (pVGC) (Poimenidou et al., 2018).

Viable *L. monocytogenes* cells enter host cells when ingested by macrophages, where the bacterium uses surface proteins internalin (InlA) and internalin B (InlB) to adhere to and invades non-phagocytic enterocytes or M cells in Peyer's patches that are

located in the small intestine (Kathariou, 2002). Host cell receptor interaction causes the host cell proteins to become phosphorylated, resulting in a signal transduction cascade that leads to pathogen-mediated internalization (Kathariou, 2002). Once *L. monocytogenes* is internalized, the bacteria excretes a pore forming protein called listeriolysin O encoded by *hly* that lyses the vacuole and can escape the cytoplasm (Kuhn and Goebel, 1999). The bacterium becomes motile within the cytoplasm of the cell host when the actin filaments from the host cell enable nucleation after conjugating with their *ActA* proteins, producing an actin tail at one pole (Kuhn and Goebel, 1999). Pseudopod-like structures are formed and the bacteria can invade neighboring cells when enveloped in a double membrane and protected by secretions of listeriolysin O and phospholipases (*plcA* and *plcB*). Invasion of major organs by dissemination of the bacteria can occur due to somatic cells such as macrophages and neutrophils and may be eliminated by macrophage and neutrophils. When T-cell mediated immunity cannot eliminate the bacteria, a systemic infection and invasion of secondary organs such as the nervous system, placenta, and fetus, occurs (Kathariou, 2002).

Several stressors can cause prfA to be expressed and enable synthesis of virulence factors (de las Heras et al., 2011). These adverse stressors include cleaners and sanitizers, refrigeration, freezing, heating, acid, salt, dehydration, and osmotic stress when persisting in food (Kathariou, 2002). This is exemplified when induction of ATR occurs after exposure to a sublethal pH level or osmotic stress (O'Driscoll, Gahan, and Hill, 1996). Mutants that have become acid tolerant also have shown survival in vivo and greater persistence. Repeated exposure to stressors such as these may create bacteria that have increased virulence (De Jesus and Whiting, 2003).

Virulence has also been demonstrated by some *L. monocytogenes* strains due to truncated internalin (Jacquet et al., 2004). Expression of the full length of internalin was correlated with nosocomial and clinical strains while isolates from foods were associated with truncated proteins. Expression of full length proteins were found in 95% of strains involved in non-pregnancy related foodborne illness cases, suggesting that the full-length internalin is necessary for bacterial transmission across the placenta (Jacquet et al. 2004). Strains with serotypes 4b and 1/2b express the full-length internalin, unlike strains with serotype 1/2c which are rarely correlated with clinical isolates. This provides epidemiological indication that full length internalin expression is essential for human listeriosis infection and truncated internalin may associate with asymptomatic carriers (Jacquet et al. 2004).

Enrichment Methods

Standard methodologies used for the detection and isolation of *Listeria* spp. from food and environmental samples are based on ISO, FDA and USDA methods. These methods require a 24 hour primary enrichment, followed by another 24 hour secondary enrichment. This allows growth to detectable levels for further growth on selective and/or differential media. Successful isolation and detection of *Listeria* spp. relies on the enrichment procedures used (Flanders et al., 1995; Pritchard and Donnelly, 1999; Sheth et al., 2018) and is exemplified later in this thesis in Chapter 4. Enrichment procedures contain nutrients and antibiotics that are meant to suppress the growth of unwanted microorganisms but will not injure or inhibit the target organism. However, research has shown that selective media, such as the University of Vermont (UVM) modified *Listeria*

Enrichment broth has hindered growth of *Listeria* spp. populations when compared to a non-selective broth such as Brain Heart Infusion (BHI) media (Bruhn, Vogel, and Gram, 2005). Antibiotics commonly added to *Listeria* spp. enrichment broths are (i) acriflavin to inhibit RNA synthesis and mitochondriogenesis of Gram-positive cocci, (ii) nalidixic acid to inhibit DNA synthesis of Gram-negative microorganisms (Beumer et al., 2009), and (iii) cyclohexamide to inhibit the growth of fungi (Pritchard and Donnelly, 1999). The FDA method requires a primary enrichment of Buffered *Listeria* Enrichment Broth (BLEB) that consists of Tryptone soy broth (30.0g), yeast extract (9.6g), monopotassium phosphate (1.35g), disodium phosphate anhydrous (9.6g) and pyruvic acid (1.11g) per liter of ddH_2O, and is autoclaved for 15 minutes at 121°C. The three aforementioned antibiotics are then added to BLEB after non-selective preincubation (4 hours) to promote repair of injured *Listeria*. Acriflavin is effective at suppressing Gram positive cocci at 40 mg/liter and allows *Listeria monocytogenes* to thrive. However, levels of acriflavin of 15 mg/liter have been shown to suppress the growth of *L. monocytogenes*. Naladixic acid levels of 20-40 mg/l also do not suppress *Listeria* spp., until levels of 100 mg/liter are reached. Acriflavin predominantly affects the 4b serotype of *L. monocytogenes* when compared to 1/2a and 2b to increased lag time. Based on the food and the concentration of acriflavin present, proteins bound between 19-79% of the inhibitor and binding is dependent on pH levels. This provides insight as to why the 4b serotype is more commonly isolated from listeriosis patients than from food products (Burall et al., 2017). Selective media seems to suppress some species within the same genus more so than others. University of Vermont (UVM) broth and Fraser broth (FB) support growth of higher populations of *L. innocua* when compared to the growth potential of *L. monocytogenes*. The FDA method of using

Buffered *Listeria* Enrichment Broth (BLEB) helps provide a buffer for the otherwise decreasing pH that occurs during enrichment with standard LEB due to glucose incorporation and other buffering limitations (Beumer et al. 1996). This decrease in pH impacts the efficacy of acriflavin and allows for overgrowth of other microflora. Fraser broth is used as part of the mUSDA and ISO methods as secondary and primary enrichment due to lessened inhibitory impact.

Use of selective media such as UVM or LRB is not effective when growing sublethally injured cells resulting from exposure to heat, cooling, drying, or after being exposed to chemicals such as acetic chloride, sodium nitrite, and sodium chloride. This inability to detect otherwise injured cells via enrichment is crucial because these damaged cells have the capability to repair in foods and regain their pathogenicity. The use of non-selective media such as Brain Heart Infusion (BHI) or Buffered Peptone Water may permit better repair and detection of injured cells (Pritchard and Donnelly, 1999). Non-selective media such as BPW is also desirable due to the buffering capacity which increases the efficacy of reviving injured cell detection regardless of the inability to inhibit other microorganisms (Walsh, Duffy, and Sheridan, 1998; Duffy et al., 2001). For the detection *L. monocytogenes* in foods with high protein contents, such as dairy products like cheese, non-selective media is more effective for short term incubation, as overgrowth of background microflora becomes more prominent in nonselective media (Walsh, Duffy, and Sheridan, 1998).

Competitive advantages between species of *Listeria* may produce false negative results in certain media (Beumer et al., 2009; Curiale and Lewus, 1994; Petran and Swanson, 1993; Ryser et al., 1996). In selective media such as UVM (Beumer et al.,

1996, Curiale and Lewus 1994, Petran and Swanson, 1993), LEB MacDonald and Sutherland, 1994), and FB (Curiale and Lewus 1994; Petran and Swanson, 1993), *L. monocytogenes* requires longer generation time periods allowing *L. innocua* to achieve a faster growth rate. *L. monocytogenes* has also seen greater lag times in non-selective media such as BHI (Evanson et al. 1991) and Trypticase Soy Broth (TSB; Curiale and Lewus, 1994). However, research has shown that *L. innocua* has no competitive advantage in minced beef samples when enriched in BPW or UVM, a non-selective and selective medium respectively (Duffy et al. 2001).

Competition between serotypes of *Listeria* spp. could also explain why serotype 1/2a is more often isolated from foods and facilities than serotype 4b, which are commonly associated with outbreaks (Bruhn et al., 2005). This phenomenon also may be attributed to genetic lineages of *L. monocytogenes* where *L. innouca* may displace lineage 1 (not lineage II) strains in UVM 1 and UVM II broths (Bruhn et al. 2005).
Lineages can also affect the growth of other bacteria during co-inoculation as it can lead to disproportional representation. At cell populations of 10^6 CFU/ml (Bruhn et al. 2005), lineage II strains outcompete lineage I in UVM II and I. Overall, this occurrence could be detrimental for epidemiological studies.

Dual Enrichment

Dual enrichment is followed by AOAC approved BAX detection methods for foods, and the USDA-FSIS method for processing of red meat, poultry, eggs and environmental samples when targeting *Listeria* spp. UVM broth is primarily used by the USDA-FSIS due to its ability to be selective with the suppression of other flora so that *Listeria* spp.

can be detected in otherwise contaminated samples. However, as discussed previously, antimicrobial agents may also impact the recovery of injured *Listeria* spp. cells (Beumer et al. 1996; Ryser et al. 1996; Busch and Donnelly, 1992). The modified USDA/FSIS method includes the use of *Listeria* Repair Broth (LRB) in addition to UVM broth to better enhance detection before aliquoting 50 µl of sample into Fraser broth. LRB is used to repair and recover inured cells as part of a 5-hour non-selective incubation period (Busch and Donnelly, 1992) prior to addition of antibiotics. Neither broth has an advantage over the other (Ryser et al. 1996) but they improve detection when used together (Flanders et al., 1995; Pritchard and Donnelly, 1999). It is also beneficial when primary enrichments are combined into a secondary enrichment broth, enabling sensitivity of the procedures individually without the extra labor (Pritchard and Donnelly, 1999). The AOAC BAX method requires the use of MOPS-BLEB broth which consists of Trypticase soy broth (30.0g), MOPS free acid (6.7g), MOPS Sodium salt (10.5g), and yeast extract 6g) per liter of ddH_2O, and is autoclaved for 15 minutes at 121°C. For BLEB, after a 4 hour preincubation period to allow repair of injured cells in the non-selective media, antibiotics are added. When the primary BLEB enrichment is aliquoted to the secondary MOPS enrichment, antibiotics are added immediately.

Agar Plating Media

After enrichment the sample is streaked onto selective media to detect the organism of interest. The FDA BAM currently recommends use of PALCAM (polymyxin-acriflavin-lithium chloride-ceftazidime-aesculinmannitol), Oxford, LPM (Lithium chloride-

phenylethanol-moxalactam with esculin and iron), MOX (Modified Oxford *Listeria*), and ALOA (Agar *Listeria* Ottavani and Agosti), Chromogenic Agar (FDA BAM, 2018).

Isolated *Listeria* spp. can be further differentiated through hemolysis and CAMP (Christie, Atkins, Munch-Peterson) tests. These tests use the byproducts of *S. aureus* and *Rhodococcus equi* to enhance the hemolysis of *Listeria* (Khelef et al., 2006). Other tests include acid production from 5% solutions of mannitol, L-rhamnose, and D-xylose (Holt et al. 1994). Acid production in L-rhamnose and not mannitol or D-xylose will determine confirmation of *L. monocytogenes* (Holt et al. 1994). Considering that methods are time consuming, chromogenic media has become a popular, less labor intensive and less time consuming method to identify *Listeria* spp. These include CHROMagar *Listeria* (CHROMagar, Paris France), BCM (Biosynth), ALOA (AES Laboratories, France), and Rapid L'MONO (Sanofi Diagnostic Pasteur, France) R&F *Listeria monocytogenes* Chromogenic Plating Medium and CHROMagar *Listeria*. (R&F Laboratories, Downers Grove, IL), and Chromogenic *Listeria* Agar (Oxoid Ltd, Basingstoke, England) have been introduced. Chromagar is a proprietary selective medium differentiates *L. monocytogenes* and *L. ivanovii* from other *Listeria* species based on these species' phospholipases. Differential activity for all *Listeria* species is due to the addition of a chromogenic substrate 5-bromo-4-chloro-3-indoxyl-β-D-glucopyranoside (Reissbrodt, 2004). These media generally differentiate between virulent strains of *Listeria* spp. (such as *L. monocytogenes* from *L ivanovii* or *L. innocua*) through activation of the lecithinase operon that contains two virulence genes actA and plcB, where plcB is responsible for encoding phosphosphatidylcholin-phospholipase C (PC-PLC). CHROMagar *Listeria* also takes advantage of β-D-glucosidase to cleave the

chromogenic substrate, producing turquoise colonies that identify *Listeria* spp. *L. monocytogenes* is identified by the formation of insoluble fatty acids that perform opaque halo zones of precipitation when L-α-phosphatidyl-inositol by PI-PLC is cleaved (Park et al., 2014; Reissbrodt, 2004).

For the purposes of this dissertation, further *Listeria* spp. isolation and identification, ChromList™ (DRG International, Springfield, NJ) is used as a selective medium. This required 51.5g of base agar to be added and dissolved into 1L of ddH_2O and is autoclaved for 15 minutes at 121°C with 15 psi. Surface plating typically requires 0.1 mL of sample onto CHROMagar™ *Listeria* and is incubated for 24 hours at 37°C. Typical *L. monocytogenes* colonies appear as blue colonies with a diameter less than 3 mm and a white halo, while *L. innocu*a appears as blue colonies without a white halo[1].

Polymerase Chain Reaction (PCR)

A genetic Polymerase Chain Reaction (PCR) can detect a pathogen in a sufficient amount of time that is shorter than standard plating tests and methods in addition enhanced detection of *L monocytogenes* that would otherwise be hindered by other *Listeria* spp. (Beumer et al. 1996; Curiale and Lewus, 1994; Petran and Swanson, 1993; Ryser et al. 1996). PCR increase the sensitivity and specificity of pathogen detection (Gasanov, Hughes, and Hansbro, 2005). PCR has the advantage of its ability to detect target organisms; such as *L. monocytogenes* while high levels (10^6-10^7 CFU/ml) of other background *Listeria* spp. may be present (Navas et al., 2006). The BAX® PCR system

[1] http://www.chromagar.com/food-water-chromagar-listeria-focus-on-listeria-species-37.html#.W9iM7CcpDUp

(Dupont™ Qualicon BAX® Q7) performs more efficiently than standard plating methods (Nortonet al. 2001b, Silbernagel et al. 2004), and has been used in the past by the USDA (MLG 8A.03;USDA/FSIS, 2008) and Health Canada (Warburton and Pagotto, 2005) to screen samples for pathogens of concern such as *L. monocytogenes*. The USDA uses the BAX PCR system to detect for *L. monocytogenes* in meat and poultry samples. This requires a dual enrichment process described previously using BLEB and MOPS-BLEB enrichment broths before using the BAX system. After enrichment, cells lysis occurs, and bacterial DNA is released where primers target a DNA sequence that is amplified for detection. This amplification is detected within the BAX, which eliminates the need for any gel electrophoresis. While the BAX method is effective and requires a reduced amount of time and is more cost effective, some foods and media ingredients (e.g., ferric ammonium chloride) may interfere with the PCR process and should be considered before moving forward as a detection method (Parameswaran, Guyer, and Knabel, 2003). BAX has been used to detect *L. monocytogenes* within environmental samples obtained from a smoked fish processing facility (Norton et al. 2001b). The PCR method has identified more positive results in samples when compare to the plating methods, discrepant results do occur based upon the low populations of pathogens which are masked by the background microbiota present. These false negative results are likely due to the low populations reached when using only a primary enrichment method instead of a dual enrichment procedure that would otherwise enable bacteria to reach levels of detection (Norton et al. 2001b). Use of a primary enrichment may also not dismiss the possibility of false positive results when amplifying DNA from cells that are not viable (Navas et al. 2006).

The Center for Disease Control and Prevention (CDC) describes how culture-independent diagnostic tests (CIDTs) are changing how clinical patients are diagnosed with foodborne illness[2]. Clinical laboratories now have the capabilities to rapidly identify the cause of an illness without needing to grow and confirm cultured isolates. This has led to a 96% increase of outbreak identification in 2017 when compared to averages from 2014-2016[3]. Examples of CIDTs are nucleic acid amplification testing (NAAT), PCR, enzyme immunoassay (EIA) and whole genome sequencing (WGS).

Molecular Subtyping

Molecular subtyping is a reliable method used to differentiate isolated *Listeria* species and variants within the same species (Wiedmann, 2002). The types of molecular subtyping include (i) multilocus enzyme electrophoresis (MEE), (ii) multi-locus virulence sequence typing (MVLST), (iii) multiple-locus variable-number tandem repeat analysis (MLVA), (iv) restriction enzyme analysis, (v) pulsed field gel electrophoresis (PFGE), (vi) single nucleotide polymorphism (SNP) genotyping, (vii) terminal fragment length polymorphism (T-RFLP) analysis, and (viii) ribotyping (Graves and Swaminathan, 2001; Schuchat et al., 1991). Researchers had concluded that ribotyping and MEE were reliable methods to use when subtyping *L. monocytogenes* strains, with the exception of serotypes 1/2b and 4b.

While WGS is the new method of identifying pathogens to determine the source

[2] https://www.cdc.gov/foodsafety/challenges/cidt.html
[3] https://www.cdc.gov/foodnet/reports/prelim-data-intro-2017.html

of outbreaks, identifying isolates with subtyping continues to be a useful tool to characterize pathogens beyond the sub-species level. Ribotyping has been previously used to link food and environmental isolates of *L. monocytogenes* with human clinical listeriosis cases (Arimi et al., 1997; Graves et al., 1994). Subtyping is an effective tool to monitor persistence and understand the ecology of pathogens (Norton et al. 2001b) within food processing facilities (Autio et al., 1999; Ho et al., 2007; Norton et al., 2001; Wulff et al., 2006) and farm environments (Arimi et al. 1997; Nightingale et al., 2004). Phenotying and molecular typing have been used to identify *L. monocytogenes* strains with high discriminatory power, typeability, reproducibility, and automation (Wagner and Allerberger, 2003). Serotyping became one of the initial phenotype methods used for identification of *L. monocytogenes*.

In regards to cheese, serotypes 4b and 1/2b were associated with listeriosis cases (Wiedmann, 2002; McLauchlin, Greenwood, and Pini, 1990) and 4b is typically associated with outbreaks (Wiedmann, 2002), where serotype 1/2a, 1/2b and 4b strains cause over 30%, 32% and 34% of listeriosis cases, respectively. This is particularly true with cheeses and cheese products (Pintado et al., 2005) and are sporadically isolated from foods (Gilbreth et al. 2005). The 1/2 serotype has been identified on dairy farms from environmental samples originating from feed, silage, and feces (Borucki et al., 2004). Serotype 1/2a strains are also isolated from ready to eat foods and food processing facilities (Kathariou, 2002). Several outbreaks were implicated by strains that were detected through WGS and were differentiated using sequence types (ST), which were formerly indistinguishable by pulsed-field gel electrophoresis (Chen et al. 2017). These ST can be traced back to epidemic clones and clonal complexes (like the caramel apple

outbreak, finding strains originating from clonal complex 1), demonstrating that these emerging clones have been diverging from its ancestors for decades.

Epidemic Clones

Epidemic clones (ECs) of *L. monocytogenes* are isolates that are genetically related, yet have been identified at a different time period or in a different geographical location (Cheng et al., 2008; Lomonaco et al., 2013). Multi-virulence locus sequence typing (MVLST) was used to identify the five original EC's associated with *L. monocytogenes* (Chen et al., 2007) and the two more recent novel ECs (Lomonaco et al. 2013). Strains isolated from the cantaloupe outbreak in September of 2011 were identified and associated with newly identified EC VI and EC VII, suggesting that these strains may colonize in specific niches within the processing facility (Lomonaco et al. 2013). Methods to identify these novel ECs included multi-locus sequence tying (MLST) and comK prophage JF sequencing, in addition to MVLST. Strains from 6 of these 7 ECs were also found in U.S. chicken processing facilities, suggesting that strains are not restricted to one food commodity.

Phage Typing

Listeria phages have been isolated from sewage plants, silage, food processing environments, and lysogenic strains (Hagens and Loessner, 2014), where 500 phages have been identified and only a select few have been genetically characterized. Large A511 phages use their cell wall peptidoglycan as a primary receptor (Habann et al., 2014), where the *luxAB* transduces a *luxAB* fusion to code for bacterial luciferase

(Loessner et al., 1996). Phage also produce cell-wall-hydrolyzing enzymes known as endolysins. They are a two-domain structure with a N terminal catalytic domain and an associating C-terminal cell wall-binding domain (CBD) that comes into contact with high affinity ligands associated with the cell wall (Loessner et al., 1995). Other transducing phages have also been used to transfer genes between strains to manipulate genetics and phenotypes (Hodgson, 2002). Commonly occurring strains in foodborne outbreaks (serovars 1/2 or 4b) are most susceptible to infection by phage and as a result can be differentiated through phage typing. While phages cannot be used to treat *L. monocytogenes* infections, prevention is possible employing anti-*Listeria* controls during and after food manufacture.

Ribotyping

Molecular subtyping techniques for discrimination of *L. monocytogenes* strains include PFGE (Brosch, Chen, and Luchansky, 1994), MEE (Graves et al. 1994) and ribotyping (Graves et al. 1994). Subtyping *Listeria* spp. became common once the Riboprinter® Microbial Characterization System by DuPont Qualicon was introduced in the mid 1990s. It is an automated subtyping system that uses restriction endonuclease EcoRI (Qualicon) or PvuII (Qualicon) DNA fragments that are modified through a modified southern hybridization blotting technique when using electrophoresis. The hybridized DNA is labeled with an *E. coli* rRNA operon probe that detects ribosomal RNA genes that are present at different points in the chromosome (Lukinmaa et al.,

2004). Images are taken by a camera that are processed using the RiboExplorer® software which situates fragment patterns that dictate band density and proximity with a normalized standard marker set. These band patterns are characterized and matched to reference patterns that are already stored in the RiboPrinter database, where bands are based upon detection of the 4, 14 and 23S ribosomes. These patterns in the database are assigned to DUP-IDs with a similarity up to >0.96 (Lukinmaa et al., 2004). A proprietary mathematical algorithm is used to determine genetic similarity of isolates according to the assigned patterns, where these patterns are then associated with a specific ribogroup (Lukinmaa et al. 2004). This can be challenging when the riboprinter has limited serotypes for certain strains and, therefore, the pattern cannot be identified. This can lead to grouping of isolates that are not similar to other isolates, decreasing discriminatory results (Lukinmaa et al., 2004).

Other challenges identified are that a DUP ID may have more than one band pattern associated, particularly when weak bands are created. The database has subsets of DUP-ID patterns that are assigned in alphabetical order which allow for comparisons with data in scientific research. When unrecognized DUP ID's are associated with specific ribotypes, the Food Microbe Tracker (formerly known as PathogenTracker 2.0) database[4] obtains RiboExplorer files to compare patterns. This allows strains to be assigned alphabetically through similar patterns that may be identified in the Food Microbe Tracker. When using EcoRI, the discriminatory power demonstrated with automated ribotyping is less than PFGE, particularly with serotype 4b strains that are

[4] www.foodmicrobetracker.com

essential for epidemiological linked in studies (Lukinmaa et al., 2004; Swaminathan et al., 2001).

This ability can be improved, when using additional restriction enzymes including PyuII (Aarnisalo et al., 2003; De Cesare et al., 2007). This automated ribotyping method was once the preferred method for larger sets of data, because it efficient, standardized, and easy to use (Lukinmaa et al. 2004). It is sensitive and can be used for comparative analyses between large data sets such as PathogenTracker 2.0. Riboprinting is still a method that can be used to identify links between foods, environments, and human or animal listeriosis (Aarnisalo et al. 2003, Lukinmaa et al. 2004) although WGS is now the standard method for epidemiological investigation. Riboprinting has also been previously used to identify and track strains that originated from farm environments (Arimi et al. 1997; Nightingale et al. 2004) and processing facilities (De Cesare et al., 2007; Ho et al., 2007; Kabuki et al., 2004; Wulff et al., 2006) such as cheese manufacturers (DeCesare et al. 2007; Ho et al. 2007) and was used to foresee categories of serotypes (De Cesare et al. 2007; Nadon et al. 2001).

Pulse Field Gel Electrophoresis (PFGE)

PFGE is a method of subtyping that compares the restriction patterns of whole genomes of bacteria. The chromosome is degraded with enzymes to select 2-25 large DNA fragments (Weidmann, 2002) and then these DNA fragments are loaded on an agarose gel and separated by size for identification. The less fragment differences there are the more related the outbreak strain may be (Lukinmaa et al., 2004), where a difference of 2-3 fragments is commonly related and a difference greater than 7 fragments is unrelated.

For *L. monocytogenes*, AscI and ApaI are commonly used restriction enzymes (Weidmann, 2002). PulseNet has a database of PFGE patterns used to discriminate *L. monocytogenes* isolates, and these PFGE patterns are used to link together food and clinical isolates (Weidmann, 2002).

Whole Genome Sequencing

Genome Trakr is the first lab network established for pathogen whole genome sequencing (WGS) testing and was first used for epidemiological purposes when FDA Center for Food Safety and Applied Nutrition (CFSAN) scientists used WGS to detect *Salmonella* spp. in a 2012 as part of a retrospective outbreak of spicy tuna sushi rolls (Allard et al., 2016). In 2014, the FDA collaborated with the National Center for Biotechnology Information (NCBI) at the National Institutes of Health, where data curation and bioinformatics are provided, and genome files are submitted to the GenBank (NCBI, 2017). This distributed network now consists of 15 federal labs, 25 state health and university labs, one U.S. lab hospital and 2 other labs in the United States in addition to 20 labs outside of the U.S. WGS is used to define and track pathogens implicated in various outbreaks caused by food and environmental sources, and link them to associated clinical isolates obtained, which has tracked and found pathogens such as *Listeria* spp. in implicated food products. Currently, genomes of over 11,000 isolates of *Listeria* spp., *Salmonella* spp., and *E. coli* are available in the public database and that number continues to increase (NCBI, 2017; Buchanan et al. 2017). WGS uses the same methods used for Pulse Field Gel Electrophoresis (PFGE) however it is also able to differentiate each strain of any pathogen. This was demonstrated in the Blue Bell ice cream *Listeria*

outbreak, where PFGE and WGS was used to further discriminate and fingerprint *Listeria monocytogenes* strains (CDC, 2015) . WGS is also deemed to be a beneficial tool because the genome of a pathogen may differ between geographical regions. This is preventive tool that can enforce compliance and is capable of identifying the source of contaminated foods that have not yet caused illness or before food product goes into commerce.

Better understanding of contamination sources can help food processors develop more effective sanitation programs that can eliminate microflora that may colonize facilities (Norton et al., 2001b) and will reduce potential cross-contamination (Wulff et al., 2006).

There are different whole genome sequencing typing protocols currently used by federal agencies investigating pathogens in foods. These protocols include the Food and Drug Administration's GenomeTrakr[5] and the protocols developed at the Centers for Disease Control and Prevention, such as the whole genome multilocus typing (wgMLST) method[6]. All these efforts have the final goal of matching food and human isolates in a more efficient and faster way to determine source attributions in outbreak investigations. These molecular methods are involving by have the promise of being the fastest method to detect pathogenic bacterial strains and reduce outbreak investigations and reduce public health exposure to identified food pathogens.

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5 https://www.fda.gov/food/foodscienceresearch/wholegenomesequencingprogramwgs/ucm363134.htm

6 https://www.cdc.gov/pulsenet/participants/international/wgs-vision.html

Sequence Types

Previously, the industry recognized isolates as Epidemic Clones that share common ancestry and have similar genetic patterns. Today, sequencing genes are used to trace *L monocytogenes* as a result of WGS methods (Gray et al. 2006; Kathariou, 2002; Tompkin, 2002). WGS allows epidemiolocal data of isolated *L. monocytogenes* strains to be collected by identifying singleton sequence types (ST) (Chen et al. 2017). Identifying isolates by ST provides limited divergence to provide signal clustering information that can be used for epidemiological investigations and other WGS analyses, in addition to establishing clonal complexes (CC) when isolates are not specified by ST (Chen et al. 2017; Lee et al. 2018). While WGS can target sequence types, like ST382 isolates that were associated with the caramel apple (0-9 SNPs) and packaged leafy greens (0-4 SNPs) cases, there is a need to further investigate the number of SNPs for epidemiologically clustered isolates associated with an outbreak before a value of genetic diversity can be established. This is demonstrated with the stone fruit outbreak that occurred in Australia in 2016, where a range of 0-41SNPs were associated with found isolates, which is more than the projected number of SNP (0-40) differences within the stone fruit clusters. Therefore, isolate diversity should be interpreted along with WGS phylogeny and epidemiological support.

Most recently, *L. monocytogenes* isolates involved in outbreaks demonstrate resistance to quaternary ammonium sanitizers (quats) and such isolates are becoming resistant to phage in temperature-dependent conditions (Buchanan et al. 2017). This creates concern as persistence or ongoing introduction into food production environments

continues to occur (Chen et al., 2017).

Three efflux systems have been acquired through gene transfer. These transposons include *Tn6188*, which is typically found in serotype 1/2a strains and conceals *qacH,* a mediating efflux that targets quat resistance. Transposon *bcrABC,* also mediates quat resistance, as well as resistance to heavy metals and triphenylmethane dyes and is found on plasmids that are accepted by strains of various serotypes and clonal groups. Quat resistance is also harbored on a chromosomal island in clonal complex 8 (CC8) and mediated by *ermB*. These genes and gene cassettes that hold quaternary ammonium resistance and phage have been acquired from other bacteria and may increase persistence in manufacturing facilities.

For example, today, *L. monocytogenes* is one of the leading concerns in the food industry due to its persistence in food facilities regardless of high cleanliness and hygiene standards. Harter et al. (2017) discovered found that some environmental strains of *L. monocytogenes* have a stress survival islet SSI-2, which consists of two genes that form a functional unit. The specific genotype that is always present in the SSI-2 sequence is ST 121 that are specialized for, and found almost exclusively in, food and food processing environments. The first gene is a transcriptional regulator, regulating the frequency and activity of the second protein when under certain stressor conditions. The second gene is a protease, an enzyme that breaks down non-functional proteins when subjected to the same stress conditions. It was observed that when the regulator is not active, the protease is not transcribed and *L. monocytogenes* has a difficult time compensating for oxidative stress, concluding that expression of these genes and the proteins that they code

are up-regulated under oxidative and alkaline stress. This SSI-2 genomic islet is not commonly found in clinical isolates and is considered a new hurdle for the food industry.

Genetic lineage

Allelic analyses were used to divide *L. monocytogenes* strains into lineages I, II, and III (Wiedmann et al. 1997). Lineages II and I are identified by PFGE and MEE as subdivisions, while lineage III is identified as a subset of *L. monocytogenes* (Nadon et al. 2001). Serovar clusters are also associated with each lineage, where lineage I serotypes groups include 1/2b, 4b, 3b, and 3c, linage II includes serotype groups 1/2a, 1/2c and 3a, and lineage III serotype groups include 4a, 4b, and 4c. Lineage II and III are similar as they both are comprised of flagellar antigens and antigen c, while lineage I is often associated with antigen *b* (Nadon et al. 2001).

Lineages seem to differentiate between host and environmental association as well as virulence (Chen et al. 2006; Gray et al. 2004; Jeffers et al. 2001; Wiedmann et al. 1997). Typically, lineage I strains are associated with isolates obtained from human clinical cases (Sauders et al. 2006) when compared to lineage II strains that are usually isolated from foods and food processing facilities (Gray et al. 2004; Norton et al. 2001a; Sauders et al. 2004; Wulff et al. 2006). Strains categorized under lineages vary in their ability to produce biofilms (Borucki et al., 2003; Djordjevic, 2002) where any variation is based upon strain, not lineage. For the purposes of this dissertation, 4b and 1/2b strains shall be further described in each lineage group.

Lineage I

Isolates that are commonly associated with human listeriosis cases and outbreaks are predominantly serotype 4b strains (Gray et al. 2004; Jeffers et al. 2001; Sauders et al. 2006; Wiedmann, 1997). Most of human epidemic isolates are grouped under lineage I (Jeffers et al. 2001; Kathariou, 2002). Lineage I strains produce larger plaques in culture assays than strains of other lineages (Gray et al. 2004; Norton et al. 2001a, Wiedmann et al. 1997. This suggests that they have a greater probability of beginning onset of disease (Gray et al. 2004). While lineage I strains are prevalent and isolated from the majority of human listeriosis cases (Gray et al. 2004), they are infrequently associated with food processing environments or food products (Gray et al. 2004; Norton et al. 2001a) as a result of a inferior ability to survive and persist in the environment (Chen et al. 2006). Lineage I ribotypes include DUP-1038B and DUP-1042B, which represented a higher percentage of human listeriosis isolates than environmental isolates (5.1%). In 1986, the CDC serotyped 144 human isolates and found that 66% of them were serotypes 1/2b and 4b and were classified as lineage I strains (Nortan et al, 2001a). Similarly, out of 1,363 strains serotyped in the United Kingdom, 74% of those isolates were also 4b and 1/2b, providing further support that lineage I strains have increased pathogenic potential (Norton et al. 2001a).

Lineage II

Lineage II isolates are most commonly found in foods such as fresh soft cheeses and soft ripened cheeses (Chen and Knabel, 2007) and in food processing facilities (Gray et al., 2004; D. M. Norton et al., 2001; Sauders et al., 2004; Wulff et al., 2006), yet few have been associated with human listeriosis cases (M Wiedmann et al., 1997; Norton et al. 2001a). It is suggested that these strains are more robust in cold temperatures (7°C) (De

Jesus and Whiting, 2003) and can exhibit better survival capabilities in the processing environment (Chen and Knabel, 2007). Strains within this lineage may be well adapted to environmental stressors due selective qualities and are potentially younger strains (Kathariou, 2002). In foods, lineage II strains are present in greater concentrations when compared to lineage I and yet have lower probability (log average) of causing human listeriosis (Chen and Knabel, 2007). This decreased ability to cause human listeriosis may be due to attenuated virulence as a result of pre-mature inlA stop codons (PMSC) (Sauders et al., 2004; Van Stelten et al., 2010) explaining why lineage II strains are underrepresented in listeriosis cases but not food (Sauders et al. 2006; Van Steltan et al. 2010). Although these strains seem to have decreased virulence and, thus, not commonly associated with clinical cases, lineage II does contain epidemic clone (EC) III (Kathariou, 2002), which are ribotypes linked to a large outbreak implicated by turkey deli meats. Other ribotypes include DUP-1039C, DUP-1042C, and DUP-1045, which are shown to persist in 50% of smoked fish processing facilities, while only isolated from 7.6% of human listeriosis clinical cases (Norton et al. 2001a). DUP-1030 was also implicated in a 1981 listeriosis outbreak that took place in Carlisle England (Jeffers et al., 2001).

Lineage III

Lineaege III strains have yet to be implicated in human listeriosis clinical cases or food outbreaks (Norton et al. 2001a; De Jesus and Whiting, 2003). These strains are frequently isolated from animals and rarely from humans (Jeffers et al. 2001; Weidmann et al. 1997), representing approximately 8% of animal isolates (Norton et al. 2001a). Lineage III strains also survive and multiply poorly in food-processing environments and during processing storage (Norton et al. 2001a). The scarcity of lineage III isolates may

shed light upon the divide of *L. monocytogenes* strains between animals and farm settings from strains found in food processing environments. The increased isolation of lineage III isolates from animals suggests that it is a result of virulence that is host-specific to non-primate mammals that limits virulence in humans (Jeffers et al. 2001). Jeffers et al. hypothesizes that lineage III strains have attenuated virulence as a result of the actA allele 3, while other researchers think that the limited exposure to humans decreases likelihood of human infection (Sauders et al. 2006). Heat inactivation studies have also found that lineage III strains are very heat labile, which would naturally result in a decreased presence in foods (Gray et al., 2004).

Outbreaks of Foodborne Listeriosis

The first major outbreak of human listeriosis associated with dairy products occurred between 1945 and 1952 (Ryser, 1999) in Halle Germany, resulting in 100 or more stillbirths (Norton and Braden, 2007). Further investigation concluded that milk from a cow that had symptoms of mastitis and stillborn infants resulted from mothers consuming the raw milk from that same cow (Norton and Braden, 2007). *L. monocytogenes* continued to be a major foodborne concern beginning in 1981 after 41 cases of listeriosis (34 perinatal and 7 adult) were reported in Canada in 1981. Of these cases, 15 perinatal and 2 adult fatalities increased the mortality rate to 41%. Investigations determined the infective vehicle to be coleslaw made from cabbage that was contaminated when sheep manure from infected sheep was used as fertilizer (Ryser, 1991). In 1983, dairy products were again implicated, causing 49 cases of listeriosis with a mortality rate of 29%. The source was pasteurized milk that was processed in Massachusetts (Fleming et al., 1985).

This was followed by an outbreak in 1985, where 142 cases of *L. monocytogenes* infection and 88 deaths occurred in Los Angeles County, California. This was an outbreak that initiated public health concerns for *L. monocytogenes* by regulatory agencies (Norton and Braden, 2007). The majority of listeriosis cases were perinatal Hispanic women (63%), although it affected 98% of those that were not pregnant but otherwise immunocompromised due to AIDS, diabetes, or cancer. Soft, unripened Mexican-style cheese (queso fresco) produced by Jalisco Mexican Products Inc. was found to be the source of infection (Ryser, 1999). Serotype 4b isolates were identified from these cheese samples. Cheeses were recalled from 26 states and samples were taken from patient's homes, supermarkets and cheeses included in the recall (Norton and Braden, 2007). Investigations concluded that the pasteurization process was inadequate, whether the milk intended for cheesemaking was improperly pasteurized due to overcapacity or there was cross contamination directly from raw milk (Norton and Braden, 2007). Following commencement of the outbreak, the FDA revised the Compliance Policy Guidelines for pathogens in dairy products (CPG 7106.08) on how to manage products that were improperly pasteurized, contaminated, or packaged in unhygienic conditions (USFDA/ORA, 2005). The FDA believed that other sporadic cases were also linked to *L. monocytogenes* causing deepening concerns for public health (Schuchat et al., 1991). The FDA implemented a "zero-tolerance" policy for *Listeria monocytogenes* in ready to eat foods where one positive sample out of two- 25 gram samples tested would render the food adulterated and "may bear or contain a poisonous or deleterious substance which may render them injurious to health" as specified by the Federal Food, Drug, and Cosmetic Act, 21 U.S.C. 342 sec. 402(a)(1) (Shank et al., 1996).

Archer (2018) touches upon this issue, stating that as *L. monocytogenes* has become more prevalent in the food supply, the question of how many cells does it require for *L. monocytogenes* to cause illness has become more relevant. The FDA had previously thought that *L. monocytogenes* was avoidable with proper sanitation and cleaning practices, specifying that its presence was the equivalent to an adulterant. The industry, however, thought the organism was not an adulterant because its presence was unavoidable. The FDA has considered implementing a limit of 100 CFU/g for foods that do not support the growth of this organism but maintain the zero-tolerance policy for foods that do. However, the stone fruit (Chen et al., 2016) and caramel outbreaks (Angelo et al., 2017) have added doubt to the adding regulatory limits (Archer et al. 2018), and the question of what is "acceptable" continues to be debated.

Listeriosis cases attributed to dairy products were also being observed internationally. Surveillance of *L. monocytogenes* by Swiss health officials was completed where isolates were identified in soft, ashed rind cheeses, Vacherin Mont D'Or, which is manufactured during the winter months within the Swiss Canton of Vaud (Ryser, 1999). Cases of listeriosis in Vaud started increasing in January of 1983. Ongoing investigations linked the cheese to infections that occurred between 1983 and 1987, where 122 cases of listeriosis, 33 of these leading to fatalities, were attributed to contaminated Vacherin Mont D'Or (Lundén, Tolvanen, and Korkeala, 2004). Investigators identified two phage types that were implicated with the cheese product.

It was also determined that *Listeria* presence was due to post-pasteurization contamination because all cheeses were pasteurized after 1983 (Norton and Braden,

2007). A common practice that may have contributed to this cross-contamination of pasteurized product was the transfer of cheese between aging caves and wooden hoops that were not disinfected before use. Half of the cellars used for affinage (the process of aging cheese) were contaminated with either one or both of the phage types, which led to replacement of equipment and implementation of a rigorous cleaning and sanitizing procedure as part of the corrective actions (Norton and Braden, 2007). Danish health authorities also isolated a phage type of *L. monocytogenes* that caused illness between March of 1989 to December of 1990 where 26 individuals were infected, and six deaths resulted. An epidemiological survey suggested that the source of infection was a Danish blue cheese, although no microbiological quantitative data ever confirmed this (Norton and Braden, 2007).

France began surveillance for the presence of *L. monocytogenes* in foods in 1987 and had confirmed listeriosis cases with a phage type that was isolated from Brie de Meaux. This French cheese was a surface mold-ripened soft cheese that was made from raw milk (Ryser, 1999). The use of PFGE allowed researchers to match food and patient isolated to better determine which batch of cheese was causing illness. This preceded another outbreak in 1995, where 33 cases of listeriosis were confirmed which resulted in 11 deaths (Lunden et al., 2004). While the same strain was isolated from other cheeses that were manufactured and ripened at the same plant, it could not be determined whether raw milk or the surrounding environment was the source of the contaminant (Ryser, 1999). The French National Research Center (NRC) had identified 14 cases of listeriosis two years later that spanned a 4 month period that implicated Pont L'Eveque, a soft

washed rind cheese that was produced in Normandy. This investigation found that *L. monocytogenes* doses exceeded 1000 CFU/gram (Ryser, 1999).

The World Health Organization assembled an informal working group in 1988 that targeted listeriosis (WHO Working Group, 1988). This group stated that *L. monocytogenes* should be considered a pathogen of concern within the environment that can be introduced and transmitted by food at any point during food manufacture and distribution. To better understand the prevalence of listeriosis and what increases risk of infection, the CDC and investigators from four other states conducted a laboratory oriented study that focused on surveillance of over 18 million U.S. residents (Schuchat et al., 1991). The investigation showed that the majority of cases were linked to soft cheese, undercooked poultry, hot dogs that were not properly re-heated or cooked, and foods that came into contact with surfaces that also came into contact with delicatessen meats (Anderson et al. 1992). The FDA and USDA FSIS both manage monitoring programs that focus on mitigating risk and cross-contamination, where the FDA focuses on dairy products and FSIS focuses on cooked and RTE meat and poultry products. In 1996, the USDA-FSIS promulgated the Pathogen Reduction-Hazard Analysis and Critical Control Point (HACCP) Systems final rule (USDA/FSIS, 1996). The goal of this program was to allow FSIS authorities inspect food production facilities to ensure that both preventive and corrective actions were being taken based on risk. Since 2011, the FDA has also adopted new regulations under the FSMA reform that focuses on preventive measures to mitigate risk.

Stringent monitoring and regulatory standards were implemented as *Listeria*

outbreaks continued to occur over time. In 1994, pasteurized chocolate milk was the culprit in an outbreak resulting in gastroenteritis and fever among individuals. The outbreak was blamed on poor hygiene practices and temperature abuse which allowed *L. monocytogenes* to grow in intact packages up to 8 to 9 log CFU/ml (Dalton et al., 1997). Between August of 1998 and January of 1999, hot dogs were contaminated with *L. monocytogenes*, resulting in 108 illnesses within 24 states, where 14 were fatal and four of them led to miscarriages or stillbirths (Graves et al. 2005).

Other ready to eat (RTE) foods were implicated in *L. monocytogenes* outbreaks such as turkey deli meats, causing 29 illnesses in 10 states during May to November of 2000, which led to 8 perinatal infections (Olsen et al., 2005; Voetsch et al., 2007).

Mexican style cheeses made in North Carolina were contaminated with *L. monocytogenes* in 2000 and caused 13 cases of listeriosis, with 11 cases involving pregnant women, resulting in 5 stillbirths. Mexican style cheeses are often implicated in outbreaks because they are homemade and sold in markets or small street vendors and are often sold illegally. This contaminated cheese was found in a patient's home and two grocery stores, and it was also found in the raw milk. The source of contamination was inconclusive due to negative test results (MacDonald et al., 2005). In 2003, another outbreak related to queso fresco cheese occurred and sickened six women. Five of these women specified that they purchased the cheese at flea markets and from vendors who were illegally selling their cheese in the U.S (Norton and Braden, 2007).

After multiple outbreaks in RTE foods, HHS and USDA issued a *Listeria* Action plan to bring awareness to consumers and producers (USDA FSIS, 2003). This also provided guidance on regulatory action and strategies such as microbial product

sampling, outbreak response, and other research (USDA FSIS, 2003). Again, an outbreak between July and November of 2002 causes 54 illnesses and 8 deaths (3 stillbirths) from contaminated turkey deli meats. *L. monocytogenes* isolates were found in a processing environment and in the product, resulting in >30 million pounds of recalled turkey deli meat. In response, the FSIS issued another document called: Directive 10240.3: "Microbial Sampling of Ready-to-Eat (RTE) Products, as part of the Verification Testing Program to assist in controlling *L. monocytogenes* through increased testing in RTE meat and poultry processing facilities (Gottlieb et al., 2006).

While regulatory actions applied between 1998 and 2008 reduced outbreaks associated with ready-to-eat (RTE) foods, red meats, and poultry, listeriosis outbreaks in dairy products are not showing the same decline (Buchanan, 2017). *L. monocytogenes* continues to be a pathogen of concern as outbreaks and recalls continue to be associated with foods that are otherwise considered "moderate" or "low" risk, such as ice cream. This was first established in March of 2015, where PulseNet databases and WGS tracked nine cases of listeriosis. Illnesses were occurring prior to that between the years of 2010 and 2014. All of the patients were hospitalized and resulted in two fatalities. This outbreak has significance particularly because of the WGS molecular subtyping methods that were used in junction with PFGE, which identified several strains[7]. Doses ranging between 8 MPN/g and 357 CFU/g were found in ice cream samples, with 99.8% of samples <100 MPN/g (Buchanan, 2017). Those who were hospitalized were served milkshakes made from the affected ice cream product. It is possible that the dose of *L.*

[7] https://www.cdc.gov/listeria/outbreaks/ice-cream-03-15/index.html

monocytogenes could have reached levels as high as 10,000 CFU/g if the milkshakes had a starting dose of 50 CFU/g and were temperature abused. This has led researchers to suggest that dietary guidelines need to address that even pasteurized products pose a risk to immunocompromised populations. This outbreak also may indicate that the standing health of the patient, immune function, and medications patients may be taking are larger factors to consider than the dose.

In 2012, Ricotta salata cheese was implicated in an *L. monocytogenes* outbreak that infected 22 individuals from 13 states and the District of Columbia (CDC, 2017a). Out of these cases, 20 individuals were hospitalized which led to 2 deaths, including one fetal loss. Subsequently, in 2013, six individuals were infected and hospitalized when an outbreak of listeriosis occurred related to cheese manufactured by Crave Brothers. One fatality was reported in Minnesota and one miscarriage.

Two outbreaks related to dairy products occurred in 2014. On March 12, 2014, it was reported that eight individuals were infected with listeriosis from implicated dairy products made by Roos Foods, where seven of them were hospitalized. One death occurred in California and five of the patients were pregnancy related. Cheese products manufactured by Oasis Brands Inc. were also associated with an outbreak that caused five cases of listeriosis, where four cases were hospitalized, three cases were pregnant women, and one death was reported.

On September 16, 2015, a recall was initiated by Karoun Dairies after distributed soft cheeses were implicated in a *Listeria monocytogenes* outbreak (CDC, 2015). Thirty people had reported being infected with *Listeria monocytogenes* since June 16, 2010, which 28 of them were hospitalized. Out of the 28 people who became ill, 21 (75%) of

those individuals reported that they had eaten soft cheese one month prior to becoming sick. Six of these illnesses were pregnancy-related, with one fetal loss. Three deaths were reported from listeriosis in California (2) and Ohio (1).

Most recently, in 2017 Vulto Creamery manufactured and distributed soft raw milk cheeses that were contaminated with *L. monocytogenes*. This outbreak caused listeriosis infection in 8 individuals who were also hospitalized. Out of the 8 individuals infected, two died and one patient was a newborn (CDC, 2017b).

FoodNet data from 2010 shows that 90% of liseriosis cases are hospitalized, twice the hospitalization fate for *E. coli* O157:H7. Out of all deaths associated with foodborne illnesses, *L. monocytogenes* infections accounts for 24%, twice as many deaths as were attributed to Campylobacter (CDC, 2011; FDA and Health Canada, 2015).

Incidence in Milk and Milk Products

The scientific literature suggests that *Listeria* spp. will more frequently contaminate processed foods rather than raw foods (Guerra, McLauchlin, and Bernardo, 2001). Other foods that are typically contaminated with *L. monocytogenes* are poultry, meat, fish, and dairy products as a result of introducing contaminated materials, cross-contamination during or after processing, distribution, or environmental contaminants.

Incidence of *L. monocytogenes* in bulk tank milk has been reported to range between 0.4 to 16% (Almeida et al., 2013; Hill et al., 2012; Jayarao et al., 2006; Jayarao and Henning, 2001; Muraoka et al., 2003; Schoder et al., 2011). The incidence of *L. monocytogenes* in European cheeses are: Italy 17.4%, Germany 9.2%, Austria 10%, and France 3.3% (Rudolf and Scherer, 2001). Soft and semi-soft cheese were most commonly

contaminated with *L. monocytogenes*, where doses found were more than 100 *L. monocytogenes* CFU/cm^2 of cheese surface and 2 samples had counts above 10^4 CFU/cm^2 cheese surface. However, Ryser (2007) reported the following contamination rates for European cheeses: Germany (4.4%), Italy (3%) and Switzerland (4.9%).

Interestingly, *L. monocytogenes* was found with more frequency in cheeses made from pasteurized milk than in cheeses made from raw milk (4.8%) (Rudolf, M. 2001). *L. monocytogenes* was present in 2.7% of raw milk samples tested in a study conducted in Ontario, Canada. Previous studies have reported prevalence rates of *L. monocytogenes* in raw milk samples that range from 1.3% in samples from Ontario, Canada to 45.3% of samples in Spain from one dairy (Steele et al., 1997).

Ryser (1991) suggests that 0.67% of pasteurized milk and 5% of frozen dairy products are implicated in listeriosis outbreaks as a consequence of post-pasteurization contamination. The FDA also reported in 1986 that *L. monocytogenes* was found in 12 of 658 samples (1.82%) of domestic cheeses tested. The year following had only one out of 181 cheese samples test positive for *L. monocytogenes*.

In addition to domestic cheeses, imported cheeses are implicated in recalls where French cheeses had less than 10% of samples contaminated with *L. monocytogenes*, while 108 out of 2425 samples (4.5%) tested positive for *L. monocytogenes* in other raw milk cheeses (Ryser, 2007). This suggests that raw milk cheeses were 5.7 times more likely to be contaminated when compared to pasteurized milk cheeses

Further investigation of the prevalence of listeriosis from soft-ripened cheeses was completed and reported in the *2015 Joint FDA/Health Canada Quantitative Assessment of the Risk of Listeriosis from soft-ripened cheese consumption in the United*

States and Canada: Report (FDA and Health Canada, 2015). According to this assessment, prevalence of *L. monocytogenes* in samples is approximately 0.6-0.7% (6-7 per 1000 servings) for pasteurized-milk cheese due to cross-contamination from the environment. The prevalence of *L. monocytogenes* in raw milk cheeses is approximately 3.2% for cheeses made in Canada and 4.7% made in the United States. The basis of this quantitative risk assessment was to accrue data to better evaluate processing and intervention strategies that mitigate contamination of *L. monocytogenes* in soft-ripened cheeses. The assessment describes that soft-ripened cheeses manufactured from raw milk poses a greater risk than soft-ripened cheeses made from pasteurized milk because the 60-day aging rule actually poses a greater risk of listeriosis.

In January of 2014, the FDA decided to testing raw milk cheeses aged for 60 days, along with sprouts and avocados, as specified in the *Summary Report: Raw Milk cheese Aged 60 Days* to better determine prevalence data and trends associated with these commodities (U.S. FDA, 2016). The FDA collected and tested 1,606 raw milk cheese samples, where 473 samples (29 percent) were domestic, and 1,133 samples (71 percent) were imported from countries such as Austria, Belgium, Bulgaria, Canada, Cyprus, Denmark, Germany, Greece, Hungary, Ireland, Lithuania, Mexico, the Netherlands, Nicaragua, Poland, Portugal, Switzerland, Turkey and the United Kingdom. It was mentioned that France and Italy are the largest exporters of cheeses to the United States. Softer cheeses with high moisture content were a priority when tested due to ability to support growth of *L. monocytogenes*. Out of all cheese samples tested, the FDA found that the contamination rate for *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* was less than one percent. Out of 1,606 samples tested, only 10 (nine semi-soft cheeses and

one hard cheese) were positive for *L. monocytogenes*. These samples were categorized into soft (fresh), semi-soft, soft-ripened, and hard raw milk cheese.

Behavior in Natural Cheese

Regulatory standards allow a variety of cheeses to be produced from raw milk according to 21 CFR 133 given that these cheeses are aged for a minimum of 60 days at temperatures of 1.67 °C (35°F) to achieve food safety. Intrinsic qualities including water activity (a_w), pH, acidity, and salt content determine the survivability of pathogens. Hard raw milk cheeses aged for 60 days meet food safety standards due to the combined factors that provide barriers for pathogenic growth (Donnelly, 2001). *L. monocytogenes* behavior in natural cheese is also based upon cell injury and the length of the lag phase. Lag time is the duration needed for cells to adjust to their new environment (Robinson et al. 1998). This is based upon the biosynthetic and homeostatic processes required for cells to adapt to the environment and undergo cell division and the period of time required to go through these processes (Robinson et al. 1998). Sodium chloride (NaCl) and pH levels limit the growth of *L. monocytogenes* by damaging cells and potential for repair, which increases the duration of the lag phase. Therefore, salt and acidic pH conditions cause microbial injury and delay the onset of the lag phase (Melo et al., 2015; Gay et al., 1996). Temperature does not have the same effect on the lag phase as pH or salt content, however. When temperatures are below 15°C with high salt content, the lag phase lengthens (Robinson et al. 1998). It has also been reported that inoculum dose impacts the duration of the lag phase and may cause cell death when stressed under unfavorable conditions (Pascual et al., 2001). With a smaller inoculum dose of *L.*

monocytogenes, cells are more exposed to stressors in ripened natural cheeses which could eliminate all cells through cell death and no growth would occur (Pascual et al., 2001).

Similar to *S. aureus* and *Salmonella* survival trends*, L. monocytogenes* has also been detected beyond the 60-day holding period for aged cheeses, where the duration of survival is dependent on hurdles such as moisture and salt content that lead to microbial injury (Gay et al. 1996). During cheesemaking, bacterial cells are entrapped within the curd matrix that results in 6-10 fold increase in concentrations contingent on cheese variety (Bachmann and Spahr, 1995; Buazzi, Johnson, and Marth, 1992; Mehta and Tatini, 1994; Ryser and Marth, 1987; Yousef and Marth, 1990; Yousef and Marth, 1988). *L. monocytogenes* can be detected after 60 days of aging when introduced as a post-processing contaminant (D'Amico and Donnelly, 2008). Populations increased with the gradual incline of pH found in surface-molded soft ripened cheeses. This is consistent with other studies showing that cheeses without the addition of a starter culture with a pH between 5.2-5.3 have promoted the survival of *L. monocytogenes*, although the organism can survive in pH levels as low at 4.4 (Larson, Johnson, and Nelson, 1999). *L. monocytogenes* tends to thrive in wet conditions, however this organism has the capability to grow when water activities are as low as 0.90 to 0.9 a_w.

Semisoft and Hard Cheeses

According to the FDA standard of identify 21 CFR 133, semisoft cheeses are defined as containing more than 39 percent, but no greater than 50 percent moisture and no less than

50 percent milkfat[8]. Hard cheeses hold similar standards with the exception of containing no higher than 39 percent moisture. Determining pathogen behavior is difficult to achieve when considering the varying manufacturing processes and physicochemical compositions. The behavior of *L. monocytogenes* during manufacture of Colby (Yousef and Marth, 1988) and Cheddar cheeses, such as reduced fat and stirred curd (Mehta and Tatini, 1994; Ryser and Marth, 1987), has been observed. As previously mentioned *L. monocytogenes* survives beyond the 60 day aging holding period, however the duration of survival is based upon moisture, salt levels, and initial inoculation levels. The growth of *L. monocytogenes* is halted when brine is introduced into the manufacture and aging process of cheese, suggesting that salt plays in important inhibitory role (Wemmenhove et al., (2014).

Several challenge studies have been conducted to better understand the fate of pathogens in cheese. When the process of cheese manufacture, such as Swiss cheeses, includes cooking of curds (typically at 50°C to 53°C) pathogens are inactivated (Buazzi et al. 1992) within 60-80 days of aging at 24°C (Buazzi et al. 1992). Challenge studies that examined the behavior of *L. monocytogenes* in Swiss cheeses made from raw milk have demonstrated that inactivation can occur within 24 hours of making cheese (Bachmann and Spahr, 1995). Parmesan cheeses or other low-moisture hard grating cheeses that require an <1-hour cooking step (~52°C), along with a small curd size and removal of moisture (whey) also achieve the inactivation of *L. monocytogenes*. If this is coupled with brining or aging, *L. monocytogenes* will become inactive and non-

[8] https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=133.187

detectable between 60-120 days (Yousef and Marth, 1990). It was also shown that *L. monocytogenes* (4-5 CFU/g) could not survive on the rind or interior of hard Italian style cheeses when introduced as a post-processing contaminant (Ryser, 1999).

While these studies are limited in more recent years, a study by (Schvartzman et al., 2011) did demonstrate that *L. monocytogenes* will not survive when making cheese from raw milk, yet pasteurized milk will support growth during cheesemaking. Within 5 hours of cheesemaking, a 2.02 log increase was observed. However, for raw milk cheese, *L. monocytogenes* increased in the core and rind by 2 log CFU/gdw within 4 days, while the pasteurized cheese did not encourage survival. This may attribute to pH as the raw milk and pasteurized cheese had a pH of 5.0 and 4.7, respectively.

L. innocua was used as a surrogate for *L. monocytogenes* in a study that observed the fate during the production and ripening of a smeared raw milk Gruyère cheese (Hammer et al., 2017). Inoculum with levels of 10^5 CFU/ml was added to raw milk and within 24 hours, levels had declined to 10^2 CFU/g. This decline was a result of high curd cooking temperatures. Core samples did not exceed levels of 10^3 CFU/g after 12 weeks of ripening and subsequently went below detectable levels after 24 weeks. On cheese surfaces, pH increased to alkaline levels during the ripening period and cheese rinds supported the growth of *Listeria* ($10^{8/}10^9$ CFU/g).

Soft Cheese

As a result of the FDA's concern on the safety of soft cheeses made from raw milk, a joint risk assessment on soft cheeses was conducted in 2015 (FDA and Health Canada, 2015). Hispanic-style cheeses such as queso fresco are dangerous to consume, however

this can be a confusing topic of discussion due to the lack of a standard of identity for categorizing soft cheeses. The FDA has conducted risk assessments for RTE foods such as fresh, soft-ripened, and soft unripened cheeses (U.S. FDA/USDA, 2003; FDA and Health Canada, 2015). It is also difficult to differentiate between Mexican-style cheeses and others as they have similar aesthetic characteristics, leading to regulatory and epidemiological concerns (MacDonald et al. 2005). Much research has been conducted to better understand the survival of *L. monocytogenes* in soft cheeses. Depending on what is used as an acidulant in the absence of a starter culture, *L monocytogenes* can survive in queso blanco styles (pH ~5.2 to 5.3) (Glass et al., 1995). It also has been shown to persist for over 90 days in cheeses such as Feta that achieve low pH and high salt contents (pH 4.6 to 5) (Papageogiou and Marth, 1989). Similar to hard cheeses, when cooking steps are introduced (57.2°C for 30 min) and accompanied with pH levels of 6.65 that are reduced to 4.7, *L. monocytogenes* will become inactive (El-Shenawy and Marth 1990; Ryser et al. 1985; Schaack and Marth, 1988). The cooking step is particularly essential for wash rinded cheeses that are also high in moisture including Camembert and Brie-types. However, according to Schnaack and Marth (1998), viable cells are also suppressed when pH levels are below 5.5. When Camembert-type cheeses were inoculated with Scott A, OH, and CA strains of *L. monocytogenes*, interior samples declined 10-1000 fold within the first 17 days of aging attributable to low pH levels and (<5.5) and storage temperature (15-16°C) (Ryser and Marth, 1987). Soft-ripened cheese made from raw goat's milk facilitated a decline in *L. monocytogenes* 28 days after manufacture to 1.5 CFU/g in the interior portion. This was attributed to low pH levels from starter culture activity (Morgan et al. 2001). *L. monocytogenes* could only be

detected by using dual enrichment methods after the soft-ripened cheeses made from milk with initial contamination levels of 10 CFU/ml. It is important to consider that heat treatment of milk can also impact the survival trends of pathogens because of eliminated natural microflora that could have otherwise have protective properties against pathogens (Donnelly, 2001).

Competitive natural flora such as *Lactobacillus delbrueckii* subsp. *bulgaricus* (*Lactobacillus bulgaricus)*, *Lactobacillus plantarum* and to a lesser extent by *Lactococcus. lactis* subsp. *lactis (Lactococcus lactis), Lactococcus lactis* subsp. *cremoris (Lactococcus cremoris)* and *Streptococcus salivarius* subsp. *thermophilus (Streptococcus thermophilus)* outcompete *L. monocytogenes* for nutrients (Pitt, 2000). Bloomy rind cheeses manufactured from raw milk had a longer lag phase to achieve at 10^3 concentrations when compared to its pasteurized milk counterpart due to thermophilic bacteria and yeast (Gay and Amgar, 2005). On the contrary, *L. monocytogenes* concentrations showed no differences after manufacture and ripening of Camembert made from raw milk and pasteurized milk (Ramsaran et al., 1998).

In certain cheeses, including Camembert styles, the presence of *Geotrichum candidum* will de-acidify the cheese surface during the aging process due to the production of a by-product called D-3-phenyllactic acid that will suppress the growth of Gram-positive and Gram-negative bacteria, inhibiting the growth of *L. monocytogenes* (Dieuleveux et al., 1998). Post-process contamination of mold-ripened cheeses, such as Camembert, is another major concern due to its susceptibility to surface contamination and the increase in pH that occurs when aging (Ryser, 1999; Ryser and Marth, 1987). Growth is typically initiated at pH levels between 5 and 6 (Ryser and Marth, 1987; Millet

et al., 2006; Ramsaran et al., 1998) with optimal growth being observed at pH levels that are within neutral to somewhat alkaline (Hammer, Bockelmann, and Hoffmann, 2017; Farber et al. 1989) as it provides a more favorable environment (Ryser and Marth, 1987) (D'Amico, 2008). Due to *L. monocytogenes*'s psychrotrophic nature, 2-3 log growth resulting in concentrations of 3-5 log CFU/g were observed with three different strains in a Camembert-type cheeses that were inoculated 10 days after manufacture and stored at 6°C and was a comparable scenario to parameters found in retail and other commercial settings (Ryser and Marth, 1987; Greenwood et al., 1991).

Survival of *L. monocytogenes* in soft and semi-soft cheeses as a result of post-process contamination showed that growth occurred at a rate of 0.5 log CFU/g in 79.2% of samples (Lahou and Uyttendaele, 2017). Various storage conditions and cheese types were noted, where soft cheeses stored at 7°C and 14°C demonstrated growth potential between 1.8-4.0 log units and 3.6-5.5 log units, respectively. Semi-soft cheeses showed lower growth potential, where an increase of 0.1-1.4 log units and 0.0-0.3 log units at storage parameters of 7°C and 14°C were observed, respectively.

In another study, *L. monocytogenes* levels of 10^3 CFU/ml were added to reduced-sodium cottage cheese with *Lactobacillus acididophilus* and *Bifidobacterium lactis* probiotics and stored at 1) 4 °C at 30% of shelf life, 2) at 4 °C at 70% shelf life, and 3) 12 °C for 28 days. In scenario 1, growth potential was between 0.5 and 0.8 log CFU/g, while scenario 2 demonstrated growth rates between 1.1 and 1.6 log CFU/g. Scenario 3 growth potential was below detectable levels (1log CFU/g). This study suggests that the addition of cultures and good hygienic practices are key to eliminate pathogen survival at low temperatures.

This scientific literature has preempted the current regulatory standards for aging since surface ripened cheeses have been implicated in listeriosis outbreaks and were associated with some of the first *Listeria* outbreaks detected.

Persistence of *Listeria* spp. in Food Environments

The ability for *L. monocytogenes* to harbor and colonize in processing plants is based upon inadequate cleaning and sanitizing, poor design of equipment or plant layout, insufficient controls in processing and/or the environment (Buchanan et al., 2017). Presence of *Listeria* has also been reported in farm, retail, and home settings, where particularly in retail environments, packaged and sliced deli meats caused 83% of all human listeriosis cases reported in the U.S. It is also important to note that the prevalence of *Listeria* in the environment is not proportional to what is typically found in foods. For example, regardless of high occurrence on dairy farms, prevalence in milk samples intended for cheesemaking could be very low (D'Amico and Donnelly 2010). Factors that play into this include herd and flock sizes, lack of milk holding for extended periods of time, seasonal milking, pasture grazing versus other sources, and implementation of sanitation standards (Buchanan et al. 2017). After *Listeria* colonizes food-processing plants, etiological and physiological traits allow the organism to persist in food products that are stored at low temperatures. Any temperature fluctuations during commercial transport and in retail allows *Listeria* to proliferate in foods as demonstrated in produce when temperatures were greater than 45°C, in 0.24% of transportation instances, 5% of back-room coolers, and 5% of display coolers (Buchanan et al. 2017;

Zeng et al., 2014). Biofilm formation within the plant environment is a leading cause of foodborne illness and likely contributes to listeriosis cases after *L. monocytogenes* strains have become established (Borucki et al., 2004; McLaughlin et al. 2011; Ryser, 1999).

Another potential mechanism that contributes to the survival of *Listeria* is the creation of persister cells (Buchanan et al., 2017). These are dormant and non-dividing cells that use long-term survival (LTS) strategy as a way to change cellular morphology from bacilli to cocci during the LTS phase. While little research has been done on this mechanism, LTS cells seem to be tolerant of temperature and high pressure. Research has shown that persistent strains of *L. monocytogenes* adhere better to surfaces such as stainless steel than other less commonly isolated strains; however conversely, studies have also found no difference in attachment or biofilm formation between sporadic and persistent strains (Ferreira et al., 2014).

It has been concluded that *L. monocytogenes* cannot be completely eradicated from processing plants because it is ubiquitous in nature and there are many entry points that can allow the organism into a facility. This requires food processors to manage trafficking and entry points and, thus, substantiates the need for preventive controls such as environmental sampling plans, good manufacturing practices, sanitation procedures that include disassembly of equipment for cleaning, and other processing measures used to eliminate RTE foods from being implicated in foodborne outbreaks as a result of post-processing contamination (USFDA, 2013).

Dairy Processing Facilities

Listeria monocytogenes persists in RTE food processing plants which includes dairy

processing and cheesemaking facilities due to a saprophytic lifestyle (Kabuki et al., 2004; Pritchard et al., 1994; Rückerl et al., 2014; Wagner et al., 1996). Dairy products have been implicated in outbreaks and sporadic foodborne illness cases overtime due to post-processing contamination (Buchanan et al., 2017; McLauchlin et al., 1990). Dairy processing facilities may contain a variety of *L. monocytogenes* strains including those that are found in clinical cases (Arimi et al., 1997) however, are considered to be the least contaminated facilities when compared to meat and poultry (Chasseignaux et al., 2001; Muhterem-Uyar et al., 2015) or fish processing (Norton et al., 2001).

In dairy processing facilities, contamination rates of *Listeria* spp. are 7.7-76.2% and 7.7-35% for *L. monocytogenes* (Fox et al. 2009; Charlton et al. 1990; Cox et al. 1989; Pritchard et al. 1994; Walker et al. 1991). There have been instances where contamination rates were as high as 100% in farmstead processing facilities, suggesting that the risk of contamination may be greater if dairy processing facilities have an external farm environment (Muhterem-Uyar et al., 2014; Pritchard et al. 1994). This provides the opportunity for dairy cattle, raw milk, and silage to enter and contaminate the processing facility (Arimi et al., 1997). This is corroborated with other studies reporting that when external farms are not present, contamination of the "outer" environment was almost zero (Muhterem-Uyar et al., 2014). Ribotyping has been used to identify many of the *L. monocytogenes* strains found in dairy processing facilities that have been linked to farm environments (Arimi et al., 1997). However, as aforementioned, rates of isolation found in farm environments (57.9%; Pritchard et al. 1994) are not proportionate with isolation rates in dairy processing facilities (D'Amico and Donnelly, 2010; Ho et al., 2007) although isolation rates from dairy processing facilities reported

have been as high as 64.5% (Charlton et al., 1990; Flanders et al. 1995; Walker et al. 1991). Risk of product contamination in dairy facilities is increased due to processes such as manufacture, hooping, cutting and packaging when compared to fluid milk. Notably, results obtained from farmstead processing plants do not reflect the extent of contamination variations between cheese production facilities (D'Amico and Donnelly, 2008).

Artisanal cheesemakers also use direct hand contact with the product during cheesemaking (Uhlich et al., 2006). Prevalence of *L. monocytogenes* in facilities that produced milk, frozen dairy products and dairy ingredients had higher rates of prevalence when compared to dairy facilities that manufactured cultured dairy products. Plant layout and design are other factors that can influence contamination rates and one study showed that larger and newer facilities had lower frequencies of contamination when compared to older facilities (Kabuki et al. 2004). Conversely, *L. monocytogenes* could be detected more frequently in larger manufacturing facilities due to personnel movements, food workflows, longer manufacturing days, and increased contact with materials, than smaller facilities (Autio et al., 1999; Rückerl et al., 2014).

L. innocua and, subsequently, *L. monocytogenes* are most frequently isolated from dairy processing facilities (Pritchard et al. 1994). Out of 705 environmental samples tested, only a 6.7% and 2.1% incidence for *Listeria* spp. and *L. monocytogenes* were reported, respectively (D'Amico and Donnelly, 2008). Another study conducted in Vermont recovered *Listeria* spp. from 57.9% of environmental samples when a farm was present, compared to 38.5% of samples when no farm was within the vicinity of the dairy processing plants (Pritchard et al., 1994). A survey administered in California found that

7.7% to 42.9% of milk samples tested positive for *Listeria* spp. depending on the type of farmstead, with cheese plants environmental testing resulting in 9.8% and 4.9% of isolates tested positive for *Listeria* spp. and *L. monocytogenes*, respectively (Charlton et al., 1990). These results are lower than some other surveys completed on a farmstead dairy facility in New York State and in Latin-style cheese processing facilities. While methodology may contribute to varied results, D'Amico and Donnelly (2008) continued to report a greater number of negative results while using similar methods. Testing for *Listeria* spp. is a good predictor of *L. monocytogenes* contamination as *Listeria* spp. have always been present when *L. monocytogenes* has been detected in processing environments. Contamination rates of singly detected *Listeria* spp. and *L. monocytogenes* reported by were 54.7% and 17%, with 11.3% of samples finding both (D'Amico and Donnelly, 2008). Comparably, frozen dairy products had an incidence of *Listeria* spp. of 66% when compared to the 44.4% incidence reported suggesting that cultured dairy products incur contamination to a lesser extent than frozen dairy products or fluid milk (D'Amico and Donnelly, 2008),. While *Listeria* spp. are often solely isolated from a large number of samples (88.7% and 94.6%), D'Amico and Donnelly (2008) only found *L. monocytogenes* joined with the detection of *Listeria* spp.

Detection rates are often dependent on methodology. Behavior of *L. monocytogenes* in the presence of *L. innocua* was observed and a study reported sensitivity to detection of 13 isolates of 4b serotypes when co-inhabiting with *Listeria* spp., potentially explaining why 4b is not commonly found in food or environmental samples Zitz et al. (2011). Also, as previously mentioned, selective agents of enrichment methods may impact the growth of *L. monocytogenes*, but not *L. innocua.* Results

showed that with a ratio of 1:1 of *L. innocua* and *L. monocytogenes*, *L. monocytogenes* was detectable. If that ratio was reduced to 1:50, *L. monocytogenes* was no longer detectable. Of all samples tested using VIDAS LDUO fluorescence measurements, *L. monocytogenes* was present in 60% of samples when *L. innocua* was also present, 17% of samples had *Listeria* spp. other than *L. monocytogenes*, and 23% had no *Listeria* spp. detection whatsoever. Culturing methods brought *L. monocytogenes* down to a 45% detection rate. Other similar studies completed in the early 1990's found that 40% of 18,000 environmental samples from RTE meat and poultry products contained *L. monocytogenes* and other *Listeria* spp. (Tompkin, 2002). However, the probability of finding samples that contain *L. monocytogenes* and other *Listeria* spp. are dependent upon the stability and ecological characteristics of each plant. Testing solely for *Listeria* spp. has advantages including faster turnaround time, greater clarity of results based upon methodology, and a lesser cost for processing. An environmental sampling program that focuses on *Listeria* spp. could be a beneficial tool for gauging *L. monocytogenes* contamination, although each positive *Listeria* spp. result must be treated as if it was *L. monocytogenes*.

Sixteen Irish Farmhouse cheesemaking facilities were sampled and tested in four categories; cheese, raw milk, processing environment, and external to processing environment (farm sites) for the presence of *L. monocytogenes* (Fox et al., 2011). Thirteen of these facilities had tested positive for *L. monocytogenes*. Out of a total of 1,590 isolates that were collected, 250 (15.7%) were identified as *L. monocytogenes*. Of these 250 positive isolates, 6.3% were associated with milk, 13.1% were associated with the processing environment and 12.3% were associated to a farm external to the

processing environment.

When considering potential environmental sources of contamination, drains seem to be a commonly contaminated area within dairy processing facilities (Ho et al., 2007; Kells and Gilmour, 2004) and may be an indicator of other contaminated sites within the facility (Charlton et al. 1990). Within processing plants, floors, coolers, and areas where pooled water accumulated tend to be other common places that are contaminated (Ho et al. 2007; Kells and Gilmour 2004). Contamination in pooled water suggests that minimizing moisture in the processing environment will control pathogen presence (Pritchard et al. 1995) considering that *L. monocytogenes* can survive in aerosols that are created when water under high pressure is applied as means of cleaning and will move the pathogen into reservoirs and niches (Spurlock and Zattola, 1991; Tompkin, 2002). Notably, contaminated raw ingredients (raw milk) are thought to be another possible source of *L. monocytogenes* contamination prior to taking processing and employee handling into consideration (Autio et al. 1999). While raw ingredient contamination can be identified in the finished food product, subtyping analyses found that not all strains identified in the finished product are not consistent to what is found in the raw milk ingredient (Norton et al., 2001). This is further supported as studies have found that contamination levels between facilities that receive raw milk in comparison to those that receive pasteurized milk had no considerable differences (Walker et al., 1990). It can be suggested that the risk of *L. monocytogenes* contamination may be greater due to the formation of biofilms rather than any (residual) liquids or waste from food product (i.e. whey) (Poimenidou et al., 2009). Specifically, for dairy processing facilities, the background microflora present in milk or custard-like products did not affect the

adherence of *L. monocytogenes* onto surfaces when present at concentrations ranging from 3.5 to 5.5 log CFU/cm^2 (Poimenidou et al. 2009). Conversely, one study also illustrated that antilisterial metabolites produced by resident bacteria may impact biofilm-producing capabilities of *L. monocytogenes* (Zhao et al., 2004).

Control of *Listeria* spp. in the Processing Plants

It is important to use materials that are non-porous and can be easily cleaned and sanitized with sanitary design recognition as essential in food processing plants (Kabuki et al., 2004). Worn equipment and porous wooden utensils or shelves have been replaced with plastic or stainless steel materials due to their durability and longevity (Ismail et al. 2016; Kusumaningrum et al. 2002). Transfer rates of *L. monocytogenes* from perforated plastics to young cheese were very low over short contact times (<2 hours). This was further evidenced when perforated plastics and glass released higher concentrations of *L. monocytogenes* than wooden counterparts.

The French agency for food environmental and occupational health safety (Anses) and the European Union Reference Laboratory for *Listeria monocytogenes* (EURL Lm) have promulgated guidelines that advise that the sampling plan should include as many samples as possible to increase the probability of *L. monocytogenes* detection (Carpentier and Cerf, 2011). It is suggested that any given area being sampled is 1,000 cm^2 or greater. However, swabbing large areas around niches may be difficult and supports the minimum recommendation of at least 100 cm^2 according to the ISO 18593. It is ideal to collect environmental swabs post- cleaning and sanitizing to validate cleaning methods but also when production is occurring to determine what harborage sites form biofilms.

Even brine solutions should be tested as means for potential *L. monocytogenes* contamination. *L. monocytogenes* can survive in brine solutions for over 200 days at 4°C (Larson et al. 1999). The addition of sodium hypochlorite at 10 to 100 ppm can inactivate the organism, although these levels may not be acceptable from a regulatory standpoint (Larson, et al., 1999).

During the 1990's, the use of wooden cutting boards was not supported because wood was thought to be a difficult surface to properly clean and sanitize and so it was recommended that plastic surfaces be used instead according to the USDA Food News for Consumers (Avait et al., 2016). Currently, the USDA supports the use of plastic or wooden surfaces[9]. Guidelines from several consumer organizations of how to properly clean cutting boards were compared, and overall, general guidance agreed that washing wooden cutting boards after each use with a cleaner, then subsequent scrubbing, rinsing with warm water, and air-drying was adequate (Aviat et al., 2016).

Cleaning and heating steps effectively decreased *L. monocytogenes* populations on inoculated wooden shelves by 4.5 $\log_{10}$ CFU/cm^2 (Ismail et al. 2017; Zangerl et al. 2010). Bacterial populations gradually increased on both plastic and wooden surfaces during the production of Kulek cheese, an acidified cheese (Dervisoglu and Yazici, 2001). Yeast and mold growth occurred on both surfaces, however only growth on wooden surfaces was statistically significant. Cheese samples that were aged on wood had more proteolytic and psychrotrophic bacteria when compared to cheeses on plastic. This demonstrated that the porosity of wood allowed more movement of air and moisture, therefore supporting

[9] https://www.fsis.usda.gov/wps/portal/fsis/topics/food-safety-education/get-answers/food-safety-fact-sheets/safe-food-handling/cutting-boards-and-food-safety

microbial growth. Mariani and colleagues (2011) studied the characteristics of *L. monocytogenes* populations on wooden boards intended for cheese aging. These heat-treated (autoclaved) or untreated boards were tested after cleaning and drying steps. Findings demonstrated that *L. monocytogenes* was significantly reduced in untreated wooden boards, while persistence and growth occurred on boards that were initially heat-treated. This suggests that there are beneficial microbial populations that have created residential biofilms that provide *Listeria* inhibiting effects that provide stable aging conditions for ripening cheeses. Galinari et al. (2014) had similar conclusions, describing how biofilms play a part in microbiological safety and in cheese ripening on wooden boards. Two of six ripening shelves tested positive for *S. aureus*, and one tested positive for *E. coli* before cheese contact. After cheeses were held on all six of the wooden boards, all cheeses tested negative for both bacteria. Interestingly, researchers noticed that when *Staphylococcus aureus* populations were high in raw milk intended for cheesemaking, greater numbers of *Staphylococcus aureus* were detectable on wooden utensils (Aviat et al., 2016). Milk microflora is the main component that determines the microflora that is found on cheese rinds and wooden surfaces intended for ripening. This establishes that microbial quality of the final cheese product is linked to the quality of the raw milk used for cheesemaking.

Persistence is a concept that is often described in studies as “repeated isolation”, “isolated on different sampling dates within 2 months”, “found repeatedly in a plant for several months and years”, or “recurrently recovered in the processing plant over a minimum of one year time period, and isolated in both processing equipment and the final product” (Carpentier and Cerf, 2011). Persistence in a food-processing environment is

identified when the same strain is isolated from the same location on more than one occasion and has the same molecular type. Resistance is defined as "a capacity for adaption and survival of microorganisms in response to recommended concentration disinfectants" (Ferreira et al., 2014). It has been thought that resistance equals persistence, this is not always the case (Ferreira et al., 2014).

Some pathogenic isolates are tolerant or resistant to killing concentrations of disinfectants and sanitizers (Carpentier and Cerf, 2011; Lourenço, Neves, and Brito, 2009). However, there is no correlation between resistance to cleaners and sanitizers and persistence of particular strains in the processing environment. This further demonstrates that, while resistance to sanitizers, such as quaternary ammonium, is being identified, biofilms are contributing to sanitizer tolerance, suggesting that such strains have to ability to form a wild-type virulence that increases based on adhesion and biofilm formation (Buchanan et al., 2017). According to Kramer (2017), *L. monocytogenes* ST6 strains may be resistant to sanitizers because they are carriers of a plasmid that hold the benzalkonium chloride tolerance gene *emrC*. Isolates that carry this gene required higher levels of benzalkonium chloride to inhibit growth and exhibit higher minimum inhibitory concentrations (MICs) of amoxicillin and gentamicin when compared to isolates that do not carry this gene. The increased resistance to disinfectants and antibiotic treatments as a result of the listerial plasmid and efflux transporter (*emrC*) carried by *L. monocytogenes* ST6 may be associated with the rise in meningitis cases in the Netherlands. Quaternary ammonium efflux pumps, like the *emrC* efflux transporter, are being found in other species, including *Staphylococcus aureus, Pseudomonas aeruginosa* and *Campylobacter* spp. and are now demonstrating associated clinical relevance.

It seems that *Listeria* can better persist in environments as part of a biofilm when compared to sporadically harboring in the environment as they possibly protect the bacteria from cleaning and sanitizing procedures (Fagerlund, 2017; Ferreira et al., 2014; Borucki et al., 2003). Biofilms are typically formed in valves, joints, gaskets, as well as small crevices or "niches" that otherwise enhances the biofilms' resistance to otherwise adverse environmental conditions (Ferreira et al., 2014). This could lead to persisting strains of *Listeria* to become part of the usual microflora within the environment when sanitation of these areas does not eliminate the organism (Norton et al., 2001). The specificity of such niches suggests that degradation of equipment from normal wear may support pathogen persistence rather than characteristics associated with the processing environment (Ferreira et al., 2014). This would imply that sanitation standard operating procedures (SSOPs) and good manufacturing practices are not working (Wulff et al. 2006). There is also potential for specific parameters in a facility to select for persisting strains while inactivating others from phage resistance, bacteriocin production or sanitizer tolerance (Kathariou, 2002).

Studies have determined prevalence of *L. monocytogenes* where plants that produced milk, frozen dairy products and dairy ingredients had higher rates of prevalence when compared to dairy facilities that manufactured culture dairy products. Plant layout and design are other factors that can influence contamination rates and one study showed that larger and newer facilities had lower frequencies of contamination when compared to older facilities (Kabuki et al. 2004). Conversely, *L. monocytogenes* could be detected more frequently in larger plants due to personnel movements, food workflows, longer manufacturing days, and increased contact with materials, than smaller facilities (Muhterem-Uyar et al., 2015). Incidence of *L. monocytogenes* ranges from 0% to 52% in

dairy manufacturing facilities in different countries (Melo and colleagues 2015) The higher incidence can be attributed to decreased compliance of employee and environmental hygiene and sanitation standards.

Floors, coolers, and areas where pooled water accumulated tend to be other common places that are contaminated within processing plants, (Ho et al., 2007; Kells and Gilmour, 2004). Contamination in pooled water suggests that minimizing moisture in the processing environment will control pathogen presence (Pritchard et al., 1994). This would help explain why *L. monocytogenes* seem to persist in biofilms that do not cohabitate with other microbial species. A co-cultured *Kocuria varians* and *L. monocytogenes* cocktail detachs more easily when contact agar is applied than the *L. monocytogenes* mono-culture counterpart (Midelet and Carpentier, 2002). This behavior was attributed to the attach of the co-cultures to each other, suggesting that cohabitating microbial communities can be more effectively cleaned than *L. monocytogenes* grown without the presence of other microcolonies (Midelet and Carpentier, 2006). This is also consistent with observations made, specifying that *L. monocytogenes* is a contaminant of processing environments that are considered "clean premises" (Carpentier and Cerf, 2011). However, there is some contrary evidence that other strains that accompany pathogens such as *L. monocytogenes* will create protective barriers against the elimination of such microbial populations when disinfecting (Carpentier and Cerf, 2011; Bremer et al. 2001).

Cleaning and sanitizing is based upon the efficacy of cleaning, efficacy of sanitizing, and the nature of the bacterial population in the harborage site. Length of cell attachment plays a role in the ability for cleaning and sanitizing to be effective (Marouani-Gadri et al., 2010). Also, surviving cells in the food-manufacturing environment are

capable of adapting to low concentrations of disinfectant. When optimal environmental parameters are added, such as temperature, moisture, pH, and nutrients from foodstuff, persistence and even growth. By the time cleaning and disinfection occurs again, the reduction of microbial population is less than the previous cleaning and sanitizing step, and thus a cycle begins of harboring of bacteria in environmental niches. This phenomenon was observed with *P. fluorescens* at 10°C and *E. coli* at 20°C, respectively, where *P. fluorescens* maintained cell density for 2-weeks, and *E. coli* populations declined after a few days (Peneau, et al., 2007) and Marouani-Gadri et al., 2010) .

An alternative explanation is that resistance to cleaning and disinfection is that niches available to *L. monocytogenes* that support the growth and persistence of those organisms and not a direct response to specific chemical cleaners, adaption of sublethal concentrations, or the number of cells that have adhered to a surface (Carpentier and Cerf, 2011). This would explain why there is so consistent association between the ability of *L. monocytogenes* to adhere to surfaces and persistence in food processing environments (Ferreira et al., 2013). Cleaners and sanitizers unable to reach niche locations could also contribute to persistence of microbes such as *L. monocytogenes* in environmental surfaces. Adaption of *L. monocytogenes* has been demonstrated with several stressors, including high levels of salt and acidity in foods and low humidity and oxygen levels in the environment (Buchanan et al. 2017). Osmotic stresses increase the resistance of *L. monocytogenes* to peroxide stressors; lethal acidic conditions create acid-adapted cells, hydrogen peroxides, ethanol and other low pH chemicals, which subsequently increase resistance to heat (Ferreira et al. 2013). The introduction of these sublethal stressors result in cross-protecting against other sources of injury (Melo et al. 2015). These adaptions

result in persisting pathogens, such as *L. monocytogenes*, after exposure to sublethal conditions that alter gene and protein expression characteristics. The beta sigma factor used by *L. monocytogenes* is the standard stress response that is activated after environmental stressors are exposed. These adaption responses include persistence under abnormal acid, oxidative stress, and carbon starved environments, and osmotolerance (Melo et al. 2015).

Disinfectants and sanitizers that are commonly used to sanitize food contact surfaces, in food processing environments such cheese manufacture include, but are not limited to, halogens, peroxide, alcohols, anhydrides, aldehydes, and quaternary ammonium compounds (QACs) (Ferreira et al 2013). Each type of sanitizer targets a different mechanism of a microbial cell in the cell membrane, thiol groups, and other cellular constituents. These include proteins, enzymes, co-enzymes, and transport pumps. There is no evidence that serotype is associated with the ability to form biofilms. Although, Djordjevic, (2002) found an association between biofilm formation and phylogeny, establishing that lineage I strains (4b and 1/2b serotypes) are more capable of forming biofilms when compared to lineage II (serotype groups 1/2a, 1/2c and 3a) and III strains (serotype groups include 4a, 4b, and 4c). However, Borucki et al. (2003) found that lineage II strains better established biofilms, demonstrating the inconsistency of pathogenic virulence and impacts on resistance and persistence in food processing environments. Strains categorized under lineages vary in their ability to produce biofilms (Borucki et al., 2003; Djordjevic et al., 2002) where any variation is based upon strain, not lineage. Lineage II and III are similar as they both are comprised of flagellar antigens and antigen c, while lineage I is often associated with antigen b (Nadon et al., 2001).

Lineages seem to differentiate between host and environmental association as well as virulence (Chen and Knabel, 2007; Gray et al., 2004). Typically, lineage I strains are associated with isolates obtained from human clinical cases when compared to lineage II strains that are often isolated from foods and food processing facilities (Gray et al. 2004; D. M. Norton et al., 2001). Lineage II isolates are most commonly found in foods such as fresh soft cheeses and soft ripened cheeses (Chen and Knabel, 2007) and in food processing facilities (Gray et al. 2004; Norton et al. 2001), yet few have been associated with foodborne epidemics (M Wiedmann et al., 1997). However, they are not often seen in cases of human listeriosis (Norton et al. 2001). These strains appear more robust in cold temperatures (7°C) (De Jesus and Whiting, 2003) and can exhibit better survival capabilities in the environment (Chen and Knabel, 2007). Strains within this lineage may be well adapted to environmental stressors due selective qualities and are potentially younger strains (Kathariou, 2002). In foods, lineage II strains are present in greater concentrations when compared to lineage I and yet have lower probability (log average) of causing human listeriosis (Chen and Knabel, 2007). This decreased ability to cause human listeriosis may be due to attenuated virulence as a result of pre-mature inlA stop codons (PMSC) (Van Stelten et al., 2010), explaining why lineage II strains are underrepresented in listeriosis cases but not food (Van Stelten, et al. 2010).

In addition to monitoring sanitation and cleanliness of the environment using cleaning, disinfecting, and sampling procedures, Larsen et al., (2014) proposes other alternative solutions that could be used to control and mitigate the formation of biofilms in food processing environments that would otherwise harbor pathogenic bacteria. These

include the use of probiotics, bacteriophages, and feed additives. However, all of these methods need to be further investigated to determine their effectiveness.

Environmental Sampling Strategies and Materials

Now that FSMA is implemented, the FDA CFSAN has made environmental sampling a requirement for most food processors as a way to establish preventive controls to mitigate presence of pathogens, particularly *L. monocytogenes* due to its enhanced fitness, difficulty to remove from environmental surfaces, and resistance to sanitizers (Poimenidou et al. 2009). Environmental sampling is a routine procedure that is also specified under the EC regulation 2073/2005 that defines microbiological criteria for foods (Lahou and Uyttendaele, 2014). Detection and elimination of pathogenic strains are mostly completed using laboratory culturing, environmental swabs or pre-moistened sponges or wipes per ISO 18593. The FDA also suggests using 3M™ or World Bioproducts© pre-moistened or dry sponge swabs to complete environmental sampling. This is ideal considering that swabbing can better remove cells from flexible and uneven surfaces that are heavily contaminated (Kusumaningrum et al. 2002). It is suggested that food producers follow the overlapping "S" technique, which follows horizontal, diagonal, and vertical strokes and then using the tip of the swab or sponge to wipe the perimeter of the area being sampled. One study found that sampling results at one-hour (T-1) after inoculation when compared to time zero (T-0) demonstrated statistically significant differences on stainless steel, rubber, and high-density polyethylene (HDPE) surfaces. In this particular study, a 3M™ sponge stick pre-moistened with Buffered Peptone Water (BPW), a 3M™ environmental swab, and a

Copan foam spatula pre-moistened with 10 mL of BPW were compared. The sponge stick, foam spatula, and environmental swabs failed to detect *L. monocytogenes* in three of 27 (11.1%), two of 27 (7.4%), and one of 27 (3.7%) surface samples, respectively when inoculated with 2 log target concentration of a *L. monocytogenes* cocktail. These data suggests no statistically significant difference between swabbing devices. The ability for the swabbing device to remove cells from environmental surfaces is crucial in addition to effectively being removed from the swab for accurate test results. Other factors to consider are the materials comprising the swabs and the pressure applied when swabbing. Therefore, it is suggested that swabbing surfaces in processing plants is the best method for determining efficacy of swabbing devices after repeated quantifiable testing under laboratory conditions. Several factors must be taken into consideration when swabbing to detect the presence of environmental pathogens. These include the ability and time required for the bacterium to adhere to a surface, the surface material and surface type, and the type of broth used for inoculation. *L. monocytogenes* detection on stainless steel surfaces has been observed when using food soils of minced tuna, cabbage and ground pork (Takanhashi et al. 2011). After two hours of drying, environmental samples were collected and all were positive for *L. monocytogenes*, suggesting that broth type or food residues may influence recovery rates due to enhanced fitness (Takanhashi et al. 2011; Kusumaningrum et al., 2002). Previous studies have also shown the survival of pathogens decline quickly when present in the environment at low concentrations.

Surface type has a tendency to impact detection of low concentrations of *L. monocytogenes* after one hour of drying (T-1) (LaHou et al 2014, Ismail et al. 2016). All swabs detected *L. monocytogenes* on all surfaces at time zero (T-0) and on rubber

surfaces at T-1. At T-1 the 3M^{TM} sponge stick did not detect *L. monocytogenes* on three out of 27 stainless steel samples (11.1%) and HDPE surfaces demonstrated a 100% recovery rate. The Copan foam spatula did not detect *L. monocytogenes* on one stainless steel (3.7%) and one HDPE surface (3.7%) out of 27 total samples, respectively at T-1. Lastly, the 3M^{TM} environmental swab did not detect *L. monocytogenes* on one of the stainless steel surfaces (3.7%) but demonstrated a 100% recovery rate for HDPE at T-1.

Surface type has a significant impact on the detection of *L. monocytogenes* where the surface structure may explain some of the variation in recovery results. Other studies have reported that surface roughness and finishes of stainless steel do not impact the recovery of *L. monocytogenes* (ref). However, the material used could influence the viability of cells, explaining the variation in recovery (Silva et al., 2008).

Notably, the state of the surface may impact the detection of pathogens. There is better recovery on wet surfaces than dry surfaces, attributed to inactivated cells when the environment is low in moisture, limiting nutrient availability. It appears that *L. monocytogenes* attachment to surfaces after drying varies by environmental materials (Norwood et al., 2001). Cellular structures such as flagella, pili, and other extracellular polysaccharides affect bacterial adhesion and survival under static conditions (Poimenidou et al. 2009). On the contrary, flagellum-mediate motility account for adhesion and biofilm formation (Lemon et al (2007). This discrepancy could be due to variation in pH, oxygen tension, and nutrient availability between studies (Poimenidou et al. 2009). All of these factors may influence the effectiveness of various swabbing devices.

Outbreaks Related to Produce

In addition to STEC, *L. monocytogenes* is another pathogen of concern in soils and produce. While most human listeriosis cases are involved in cross-contamination of finished product from the processing environment, *L. monocytogenes* has been attributed to some large foodborne outbreaks. The most common food commodities contaminated with *L. monocytogenes* are raw meat, raw milk, and raw produce. Blenden and Szatalowicz (1967) reported 731 human listeriosis cases between 1933 and 1966 within the United States, but were unable to determine what cases were linked to produce. There was an outbreak related to 23 cases of listeriosis associated with tomatoes, lettuce, and raw celery at eight Boston hospitals in 1979 (Ho and colleagues (1986). In 1981, the consumption of coleslaw that was harvested from fields that were amended with untreated sheep manure caused an outbreak in Nova Scotia that caused 42 human listeriosis cases (Nightingale et al., 2004; Schlech, 1983). Two sheep had died from *L. monocytogenes* infection in 1979 and 1981. During investigation, two packages of coleslaw tested positive for *L. monocytogenes* serotype 4b. Packaging was a factor in contamination when the cabbage was kept in cold-storage between October through the winter and early spring.

In 2011, contaminated "Rocky Ford" cantaloupes from Jensen Farms located in the southeastern Colorado were implicated in one of the largest foodborne outbreaks that the U.S. has witnessed in almost ninety nine years (Nyarko, 2017; CDC, 2011). This was the first time in history that cantaloupes were a vector for a listeriosis outbreak that caused 147 cases of illness, 33 deaths, and 1 miscarriage in 28 states (McCollum et al., 2013).

Most recent produce outbreaks have also been associated with *L. monocytogenes* contamination. In 2016, eleven frozen vegetable products were recalled by CRF Frozen foods due to potential *Listeria* contamination[10]. This recall expanded to include all frozen vegetable and fruit products processed in its Pasco, Washington facility since 2014. In 4 states, a total of nine people were infected and hospitalized with the strains of *Listeria* associated with the outbreak, resulting in 1 death.

On January 27, 2016, Dole also initiated a voluntary recall on all salad mixes produced in their Springfield, Ohio processing facility due to *L. monocytogenes* contamination (USFDA, 2016). This outbreak had infected 19 people in nine states, one being a pregnant woman. One individual died from listeriosis.

Another outbreak occurred in 2015, where Bidart Bros. of Bakersfield, California initiated a recall on Granny Smith and Gala apples due to *L. monocytogenes* contamination. Environmental testing of the processing facility revealed the presence of *L. monocytogenes*. and whole genome sequencing showed that these isolates were related to the outbreak strains. Recalls on prepackaged caramel apples were initiated by Happy Apples, California Snack Foods, and Merb's Candies during this investigation,. This outbreak infected 35 people in 12 states. Of these individuals, 34 were hospitalized, and three of the seven deaths were due to listeriosis. Three cases of meningitis occurred in children ages 5 to 15 years and eleven illnesses were pregnancy-related**.**

Behavior Associated with Produce

[10] https://www.cdc.gov/listeria/outbreaks/frozen-vegetables-05-16/index.html

L. monocytogenes is commonly found on plant tissues, like raw fruits and vegetables as a result of environmental contact (Harris et al., 2003). *L. monocytogenes* has been found in environmental sources such as soil, agricultural irrigation sources, cull piles, and within food processing facilities including decaying plant residue in bins or on processing equipment. *L. monocytogenes* tends to survive longer in moist and organic soils versus dry and low organic soils (Buchanan et al. 2017). The ability of *L. monocytogenes* to persist on plant tissues and surfaces is dependent on the integrity of the epidermal barrier. Injury through bruising or tearing of the surfaces affords organisms access to the internal tissues. These injured tissues are able to better nourish microbial growth. Discovery of what affects the organism's presence or persistence has yet to be determined, however plant tissues are considered a common vector between natural environment and dissemination into the food supply. These avenues can be indirect, such as contamination of raw milk from silage use, or directly from cross-contamination. *L. monocytogenes* can survive on fresh produce when stored at refrigeration temperatures (Harris et al. 2003). This is evidenced by detection on cut fruit and vegetables such as asparagus, broccoli, butternut squash, coleslaw and cauliflower, rutabaga stored at 4°C, lettuce held at 5°C, and chicory and endives at 6.5 °C. However, produce such as carrots produce defense compounds called phytoalexins that attribute antimicrobial properties and inhibit the growth of *L. monocytogenes*. Addition of these antimicrobial compounds to other foods does not provide the same effect. When apple juice (pH 3.78) and apple raspberry juice blends (pH 3.78) were tested for the presence of *E coli* O157:H7, *Salmonella*, and *L. monocytogenes*, only *L. monocytogenes* was isolated from 50 juice samples tested. Routes of contamination often originate from non-potable water sources, presence of

cattle, deer and, in rare cases, amphibians. Out of 5 orange juice outbreaks documented, three of them were associated with contamination from infected handlers who came into direct contact with the juice commodity during preparation. Other origins of contamination were linked to water sources.

Escherichia coli

Escherichia coli are Gram-negative, facultatively anaerobic rod-shaped bacteria (1.1-1.5 mm in diameter, and 2-6 mm in length) that are part of the *Enterobacteriaceae* family (Baker et al., 2016). Most *E. coli* bacteria are considered generic (non-toxigenic) and are commonly found as commensal organisms within the gastro-intestinal tracts of mammalian hosts and environmental reservoirs, such as water and soils (Baker et al., 2016; Martin et al., 2016). They are commonly used as indicator organisms to determine post-process contamination in ready-to-eat (RTE) foods and hygienic conditions of water sources (FDA BAM, 2002). RTE foods, like cheeses, are consumed raw, or are handled, processed, mixed, or cooked without application of other bactericidal processes (Buchanan et al., 2017).

Pathotypes

Shiga toxin-producing *Escherichia coli* (STEC), also known as Enterohemorrhagic *E coli* (EHEC) or verocytotoxin producing *E. coli* (VTEC) (i.e., *E. coli* O157:H7), are one of the six known strains of pathogenic and toxigenic *Escherichia coli* in addition to (i) Enteroinvasive *E. coli* (EIEC), (ii) Enteropathogenic *E coli* (EPEC), and (iii) Enteroaggregative *E coli* EaggEC (Farrokh et al. 2013;USFDA, 2012; Kaper and Sperandio, 2005; Nataro and Kaper, 1998). Extraintestinal *E. coli* (ExPEC), is a group of

pathogenic *E. coli* that includes Avian pathogenic *E. coli* (APEC), uropathogenic *E. coli* (UPEC), and other *E. coli* that can cause neonatal meningitis and septicemia (Markland et al., 2013; Nataro, 1998). *E. coli* strains are serotyped based on three major surface antigens: (i) K (capsular), (ii) O (somatic), and (iii) H (flagellar). These serotypes are established under serogroups that are used to identify virulence factors through chromosomal markers (Nataro, 1998). EIEC is an invasive strain that causes disease in the colon by penetrating and multiplying within intestinal epithelial cell lining, resulting in diarrhea that contains blood, polymorphonuclear leukocytes, and mucus (Padhye and Doyle, 1992). EPEC causes diarrheal diseases in infants and children by attaching to the brush border microvilli of the intestines and causing attaching-effacing lesions (AE) (Law et al., 2000). EAggEC forms clumps on the epithelial cells of the intestinal lining. DAEC can adhere over the entire surface of intestinal epithelial cells. Symptoms of DAEC infections are often found in malnourished and immunocompromised children. Enterotoxin and cytotoxin are thought to cause diarrhea in young children (Padhye and Doyle 1992) and EAggEC are becoming a recognized cause for diarrhea in adults (Kaper and Sperandio, 2005).

E. coli **Phylogenic Clades**

E. coli as indicator organisms have been associated with various clades to determine source of contamination. While the FDA supports use of *E. coli* to indicate presence of filth (U.S. FDA, 2002; Paruch and Mæhlum, 2012), certain lineages of *Escherichia* are not associated with fecal sources, yet are undifferentiated from *E. coli* (Luo et al., 2011; Oh et al., 2012; Walk et al., 2009). Therefore, the use of coliforms and

E. coli as indicators to determine presence of pathogens is being challenged (Stevens et al., 2003; Wu et al, 2011) in foods such as cheese. Given that housekeeping genes of *Escherichia* phylogenic clades III, IV, and V have been isolated from freshwater beaches suggests that mutation occurs in the environment outside of the mammal gastrointestinal tract (Walk et al., 2009). Given the high risk of false positives, using *E. coli* as a hygiene indicator for raw milk cheeses is debatable (International Dairy Federation, 2016).

Recent outbreaks associated with fresh produce have been associated with *E. coli* O157:H7 strains that were linked with more severe diseases including hemolytic uremic syndrome and more hospitalizations. This suggests that an evolution resulting in increased virulence occurred (Manning et al., 2008). Researchers tested this theory by detecting SNPs in 96 loci from 83 O157 genes that were categorized into 9 clades in over 500 *E. coli* O157:H7 strains. Out of the 96 loci, 68 sites were found by genome microarrays, 15 were housekeeping genes, four were shared between two O157 genomes, with none of the sites originating from three virulence genes of *eae*, *espA*, and *fimA*. Real time PCR was used to identify mutations as SNP's for population genetic and phylogenetic analyses. Variation was identified between clades depending on the frequency and distribution of Shiga toxin genes and the description of the clinical disease. The *stx1* gene was commonly found in clade 2 strains (95.1%) but not in clade 8. Meanwhile, the *stx2* gene was found in all clades (98.5%) but was most commonly found in clade 2 (46.8% of 519 strains) and clade 8 (25.4%) strains. Clade 2 and 8 strains had the *stx2* gene in 98.4% and 100% of the 519 tested *E. coli* strains respectively. The *stx2c* gene was also found in clades 4,6,7, and 8 but not in 1,2, or 3. The sequencing of a 2006 spinach outbreak strain suggested that the clade 8 lineages have acquired virulent genetic components. Clade 8 strains tend to carry genetic

material that includes the *stx2c* and Stx2c lysogenic bacteriophage 2851, providing the strain with an opportunity to alter its genetic composition when compared to strains in other clades. Out of 333 patients from Michigan with laboratory confirmed O157 infections, those infected with clade 8 strains typically were younger (0-18) and those with HUS have a seven times greater risk of being infected with a clade 8 strain when compared to patients infected with clade from 1-7 combined. Only three HUS patients had infections originating from clade 2, where data showed that men were more commonly infected with clade 2 strains than women. Researchers also found that clade 7 strains were associated with less acute symptoms such as bloody diarrhea. Screening performed during this study also led to the conclusion that clade 8 strains were implicated in outbreaks as far back as 1984 on many continents where it was determined that the *stx2* gene and *stx2c* genes have not been reported in recent years. This still needs more investigation to determine causes for the increased virulence and transmission of O157:H7 strains (Zhang et al., 2007).

Shiga toxin-producing *E. coli* (STEC)

STEC are characterized by their ability to produce shiga-like toxins (Stx), commonly known as Stx1 and Stx2 along with their subtypes (Markland et al., 2013). STEC is named after the shiga-toxin due to the similarity of shiga-toxin created by the stx 1 gene of *Shigella dysenteriae* (Baker et al., 2016). Currently, the known subtypes of shiga-toxins produce by STEC are Stx1a, Stx1c and Stx1d, and Stx2a, Stx2b, Stx2c, Stx2d, Stx2e, Stx2f and Stx2g[11]. These subtypes are often found in strains that are more persistent in environmental

[11] https://www.fsis.usda.gov/wps/portal/fsis/topics/data-collection-and-reports/nacmcf/current-subcommittees/nacmcf-subcommittee-stec-2015-2017

or mammalian sources. The scientific literature states that Stx1a, Stx2a, Stx2c and Stx2d are most commonly isolated from cases of human illness and over 400 serotypes have been identified that can produce any of the Stx1 or Stx2 and their subtypes or a combination thereof (Farrokh et al., 2013). However, only around 100 of these subtypes are known to cause illness and many of them are not pathogenic unless other virulence factors, such as intimin adhesin, are present.

Pathogenic STEC serotypes have been found in a variety of foods, such as meats, produce, and RTE dairy products, causing concern for cheesemakers (Farrokh et al. 2013). The FDA has specified that there is currently a total of 11 STEC serogroups that have been associated with human illness: O26, O45, O91, O103, O104, O111, O113, O121, O128, O145, and O157 (FDA BAM, 2017). While O15:H7 has the highest incidence of infection due to high virulence and the ability to cause infection at low doses (5-50 cells), other variants of serotypes that were identified and associated with hemorrhagic colitis (HC) or hemolytic uremic syndrome (HUS) development include what are commonly known as the "big six": O26:H11, O111, O103: H2, O121, O145 and O45, in addition to O2:H5, and O157:NM (non-motile) (USDA-FSIS, 2016; Farrokh et al. 2013; Su and Brandt, 1995). STECS of other serogroups (non-O157) are implicated in over 60% of illnesses (112,000 cases) in the U.S. alone (Lin et al., n.d.). These non-O157 serogroups: O26, O45, O103, O111, O121, and O145, are associated with over 74.2% of infections in the U.S. Specifically, serogroups O26, O103, O111, O121, and O145 have resulted in HC and HUS in infected individuals, while O45 has only been linked to HC. Serogroups O91, O113, and O128 are less frequently isolated but may hold virulence and also be causative agents of HUS and HC (Lin et al. n.d). These serotypes require the production of intimin to become

pathogenic since virulence of STEC is multifactorial and requires adhesion to the intestinal lining before toxin is released (Baker et al. 2016).

The *eae* gene is used by STEC to code for intimin, allowing intestinal invasion and attachment of STEC to the gastrointestinal tract wall of human hosts (Baker et al. 2016; Nataro and Kaper, 1998). Once inside the host, STEC will release the shiga-like toxins, commonly causing diarrhea, which is reported in 40-70% of human cases (Baker et al. 2017). Symptoms usually begin 3 to 4 days after being exposed and can last anywhere between 1 to 9 days after an infectious dose between 10 and 100 cells is ingested (Baker et al. 2016; USFDA, 2012). Once toxin is released into the gut lumen, symptoms include HC, adhering-effacing lesions, severe abdominal pain, bloody diarrhea, and severe life-threatening sequelae, such as hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenia purpura (TTP). HC is described as a quick onset of painful abdominal cramps followed by watery and bloody diarrhea (Padhye and Doyle, 1992). Approximately 3% to 7% of HC cases progress to HUS or TTP (USFDA, 2012), where only 5% of all STEC cases progress to HUS, typically 3 days after diarrheal symptoms show. This is especially concerning for those who are immunocompromised, such as children, the elderly, or those who are undergoing medical treatment, as they have the greatest susceptibility (Baker et al., 2016; Westerholt et al., 2003). Those whose disease progress to HUS can succumb to renal failure, hemolytic anemia, and thrombocytopenia. This occurs when capillaries of the kidneys and other organs are blocked due to the buildup of waste products when the endothelial damage triggers a clotting mechanism. The mortality rate due to HUS complications is anywhere from 3% to 5% (USFDA, 2012). While severe complications can occur from STEC infection, many STEC associated outbreaks go unreported due to mild

symptoms associated with illness. Also, some *E. coli* O157 isolates from human and bovine hosts are distributed differentially and may be less virulent for humans or cannot be properly transmitted from bovine sources to humans (D'Amico and Donnelly, 2010).

Evolution of Virulence Genes

The shiga-like toxins (Stx 1 and Stx 2) are A-B toxins that modify the 28S rRNA strand and through this modification, inhibit protein translation. Stx1 and Stx2 are encoded on bacteriophage and are transferrable to other cells and were originally recognized for the cytotoxicity towards Vero tissues culture cells found in the kidneys of African green monkeys (Doyle, 1991). STEC will produce these toxins after it has attached to the epithelial lining through the "locus for enterocyte effacement" (LEE) mechanism (Nataro and Kaper, 1998). The Stx 2 gene is known to be more virulent than Stx1 (Sharma et al., 2011). This is applicable to the cheesemaking industry as some studies reported that STEC isolates found in dairy products only carried the stx1 gene (Pradel, Bertin, Martin, and Livrelli, 2008). However, other studies debunk this finding (Zweifel et al., 2010). The ability for STEC to cause disease is dependent on the bacteria's ability to invade and attach to the gastrointestinal lining of the host. The *eae* gene is a major component for invading, colonizing, and attaching to the intestinal wall. Sharma et al. (2011) found a that greater colonization potential on lettuce was a result of the increased expression of *eae* in ambient air conditions. *E. coli* O157:H7 without this gene did not have any colonization potential in young rabbits, resulting in no related symptoms. Attachment genes encoded by *iha* and *rfbE* that allowed *E. coli* O157:H7 persistence were also up-regulated in lettuce packaged in ambient air conditions at 4°C and 15°C. This up-regulation of *iha* is also observed in

bovine hosts when compared to human hosts who are infected, however up-regulation of *rfbE* is observed in humans who are infected when compared to bovine models. However, *E. coli* O157:H7 does not persist in the intestines of mice (streptomycin-treated) when *rfbE* is removed, demonstrating that *rfbE* may also be necessary for attachment and pathogenesis. LEE is a pathogenicity island that encodes for a protein called *intimin*, an outer membrane protein that enables bacterial attachment to epithelial cells. This LEE also uses the *tir* gene to encode for the *Tir* protein, which is an intimin receptor, needed for cellular attachment (Nataro and Kaper, 1998). It is reasonable to speculate that *E. coli* O157:H7 does not enter the blood stream because it never causes fever. Reasoning behind why HUS can progress through STEC infection is due to the high number of Stx receptors on the kidneys and are a common site of damage from Stx toxins. Toxin molecules can be internalized by receptor mediated endocytosis and degraded by lysosomes (Law, 2000). However, other cells may process the toxin through the golgi apparatus and endoplasmic reticulum. This results the formation of a glycosidic subunit that inhibits the cell's ability to synthesize proteins by inhibiting peptide chain elongation and causes cell death (Law, 2000). According to Markland et al., (2013) the *stx* gene may be transferable to nonpathogenic *E. coli* strains through transduction and is becoming a public health concern, however many *E. coli* have *stx* genes are still not pathogenic (Farrokh et al. 2013; Kaper and Sperandio, 2005). STEC strains also encode enteroheamolysin (Ehx) with the *ehx* operon (Sharma et al. 2011). The expression of *ehx*A on lettuce was up-regulated after 10 days in storage at 15°C with ambient air conditions (Sharma et al. 2011). While the exact role of this gene needs further investigation, as it is not needed for bacterial colonization or attachment, it was observed that greater expression was observed in humans

who were infected than bovine hosts. Production of hemolysin may be a required precursor for the lysis of erythrocytes that are present when bowel mucosa and blood vessels are damaged. Free hemoglobin from lysed erythrocytes may also contribute to STEC growth and toxin production as an additional iron source (Law, 2000).

STEC O157:H7 may have the ability to induce an adaptive tolerance response (ATR) when exposed to slightly acidic conditions but induce resistance in response to exposure of more acidic conditions (Jordan and Davies, (2001). This suggests that the mildly acidic environment often found in cheeses may allow STEC to survive in the acidic contents of the stomach and cause infection at a decreased dose (Maher et al. , 2001). According to Baker et al. (2016), STEC can optimally grow between pH ranges of 5-9, but can survive pH levels as low as 2 for short durations of time. Research has also shown that exposure of certain antibiotics not only results in increased toxin production, but also leads to mobilization of phage (Zhang et al., 2000). Pathogenic STEC are hardier and therefore are able to survive lower temperatures (<15 °C) when compared to non-pathogenic *E. coli* strains (Vidovic et al., 2011; Sharma et al. 2011). Other environmental stressors can also cross-protect and lead to up-regulation of heat resistance genes that may lead to thermotolerance (Murano and Pierson, 1992).

Geographical Trends

A meta-analysis of recent outbreaks and illnesses reveals regional variations. For example, the STEC O157 serogroup is more frequently isolated from infected individuals in both island nations of the United Kingdom (UK) and Ireland (Farrokh et al. 2013). Conversely, non-O157 STEC serogroups were isolated from clinical cases in continental European

countries.

Sources of STEC

Fecal Transmission from Ruminants

STEC excretion in feces can contaminate milk intended for cheese making by direct or indirect routes. Indirectly, fecal matter can contaminate the environment through water runoff and pests or can be displaced onto teats prior to milking (Farrokh et al. 2013). *E. coli* O157:H7 has the ability to survive in soils amended with manure over long periods of time (Fremaux et al., 2008a, 2008b). Prevalence of STEC in dairy cows can range from 0-71%, with prevalence within herds ranging from 0-100% (Farrokh et al. 2013; Hussein and Sakuma, 2005). Viable cells have been found in 3-8% of dairy herds and only 0.5-1% of bovine animals. On average, only 2.2% (ranging 0% to 30%) of fecal material from 5,368 dairy animals tested positive for STEC, with an average of 0.5% of samples testing positive for STEC O157 (ranging 0% to 7.2%) (cite the EFSA 2009 report). There is no correlation between the presence of *E. coli* O157:H7 on a dairy farm and the presence in STEC in raw milk (Wells et al. 1991). However, due to variation of data collection and results, dairy producers should always be considering the possibility of STEC presence on dairy farms (Hancock et al., 2001).

Mammary Excretion from the Udder

It is speculated that STEC is in the mammary gland when a mastitis infection is present (Fremaux et al., 2006). STEC was found in 3% of milk samples obtained from cows with

E. coli mastitis in Switzerland (Farrokh et al. 2013). A study in Brazil also tested 2,144 milk samples from cows with pre-clinical mastitis and detected the *stx* gene in 12% of 182 STEC isolated strains (Lira, Macedo, and Marin, 2004). Conversely, all mastitis cases in France confirmed from recent testing to 20 years prior was caused by *E. coli* strains that did not have the *stx* gene, suggesting that colonization is not fully understood.

Methods for detection of STEC in Foods: PCR Methods

The PCR method is able to detect the +93 single nucleotide polymorphism that is located in the *uidA* gene and is responsible for encoding the identifying β-D-glucuronidase (GUD) enzyme found in *E. coli*, in addition to *stx1* and *stx2* genes, by using primers specific for these genes (Jinneman et al., 2003; Yoshitomi et al., 2003; FDA BAM Chapter 4A). The AB7500 assay has been validated for use on many foods and has replaced the LightCycler method, due to higher throughput (faster) analytical testing capabilities (FDA BAM Chapter 4A). However, this multiplex testing method is prone to error, generating false negatives or very late positives when testing mixed culture enrichment samples from foods or the environment as targets are detected from other strains, such as generic *E. coli*. Although generic *E. coli* does not have the +93 SNP, it does have the +93 *uidA* probe, which includes the *uidA* gene, and subsequently can be amplified and detected by the PCR primers, giving a negative result. Conversely, positive samples can be produced due to enrichment methods and, hence, all positive samples must be streaked on selective media to identify and confirm STEC constituents (*stx* and O157) are identified in the same isolate. These confirmatory

methods include streaking onto selective and differential agars to identify and isolate STEC or non-STEC strains for further subsequent biochemical, serological or genetic testing.

Other current rapid detection methods include Pulsed Field Gel Electrophoresis (PFGE) and whole genome sequencing (WGS). Data is submitted to programs such as Pulse Net (PFGE) or GenomeTrakr (WGS) for health risk analysis (USFDA BAM Chapter 4A).

Culture Methods for the Detection of STEC in Foods

Overnight samples of initial enrichment (18-24 hours) are serially diluted into BPW using spread-plate dilutions (FDA BAM, 2017). Typically, 0.05 mL of 10^{-2} and 10^{-4} dilutions should produce 100-300 colonies that are well isolated on petri plates. Duplicate plating onto TC-SMAC (Tellurite Cefixime – MacConkey with Sorbitol) and one additional chromogenic agar (Rainbow® Agar O157 or R&F® *E. coli* O157:H7 agar) is performed. A streak for isolation may also be completed to confirm colony morphology. These plates are incubated for 18-24 hours at 37°C ± 1°C. TC-SMAC colonies are defined as "colorless or neutral/gray with a smoky center and 1-2 mm in diameter". Rainbow® Agar O157 or R&F® *E. coli* O157:H7 agar produce black to blue-blackish *E. coli* O157H7 colonies. Other options include CHROM-agar (mauve), SHIBAM (STEC heart infusion washed blood agar with Mitomycin-C) (white colonies with hemolysis zone), or L-EMB (Levine's Eosin-Methylene Blue) (dark purple centered colonies with or without a greenish gloss). Disadvantages of cultural methods include cells becoming inactive so that they cannot be detected. Therefore, viable not non-culturable (VBNC) methods are necessary for detection in foods and environmental samples (Farrokh et al., 2013).

Molecular Methods: High Throughput Sequencing for Detection of STEC

Next generation sequencing (NGS) assays are becoming more innovative as methods to better detect and differentiate between STEC at the gene level that do and do not pose risk to human health is of utmost importance. This ability to distinguish pathogenic STEC from foods is a current concern and ongoing challenge for regulatory agencies, internationally (Carter et al. 2016). Prior to NGS, identification of *stx1*, *stx2*, and the *iudA* gene targets was determined simply using PCR SmartCylcer II and AB 7500 Fast Technology to detect STEC (Feng and Lampel, 2016). Precision of PRC is only as good as the specificity of the primers for the gene region of interest.

Most recently the BAM has been updated and includes a new method known as the "13-plex STEC Molecular Serotyping and Virulence Profiling Protocol, FDA, 2015**;** Luminex-based Suspension Array to Identify STEC O serogroups O26, O45, O91, O103, O104, O111, O113, O121, O128, O145, O157 (FDA BAM, 2017) that has been validated and approved by the FDA. This method has the ability to identify the eleven STEC O serogroups that are most commonly associated with foodborne illness in humans as well as two known *eae* and *aggR* virulent attachment genes that are expressed and allow STEC to have virulence (Lin et al., n.d.). This can help specify and distinguish pathogenic STECS from other non-pathogenic STECS that are not connected to human foodborne illness cases and identify those that are not frequently seen in food outbreaks and illness (FDA BAM, 2017). This new method shows promise because the Luminex suspension array yields accurate results. The method accurately identified all 114 STEC isolates, including non-STEC as negative (Lin et al., 2011). An independent validation yielded a 99.4% accuracy rate (Lin et al., 2013).

While NGS assays may improve identification and characterization of STEC isolates, the cost remains a deterent (Carter et al., 2016). It is more cost-effective to use agglutination methods with a single-plex O157 assay first, followed by subsequent identification of non-O157 strains using a more costly multiplex assay (Carter et al., 2016). For example, while STEC serogroups that were included in the "big six" can be isolated from produce (especially spinach and cilantro), many of these STEC lack virulence factors necessary to cause illness. Since produce have a limited shelf life of approximately 2 weeks (USDA, 2016), the Luminex method could be too time-consuming to provide accurate results that differentiate between STEC and that do and do not pose health concerns, and at the cost of the food producer, and possibly the consumer. Further, *stx* and *aggR* genes are poor indicators of STEC contamination, as *stx* genes are present in non-pathogenic STEC and may be detected without the bacterium or may be deposited through bacteriophage. A viable culture is essential to confirm STEC presence; otherwise detection of genes is not a useful method to measure health risk (EFSA, 2009).

Rifampicin resistant (Rif^R) Generic *E. coli*

Rifampicin is a broad-spectrum antibiotic that inhibits the DNA-dependent RNA synthesis by binding to the bacterial RNA polymerase holoenzyme (RNAP) which prevents elongation of the nascent RNA strand (Campbell and Gibbard, 1944; Hammerling et al., 2016). Mutations occur within the *rpoB* gene that encodes the β-subunit of RNAP, which can grant Rif^R by disrupting the hydrogen-bonding network that stabilizes Rif binding or by sterically occluding its binding site. Rif^R is conferred by single-base mutations that are always in the essential *rpoB* gene. This can be achieved

through genetic code expansion, where a codon is recoded to a 21st nonstandard amino acid (nsAA) and can either 1) create a new dimension or property to construct proteins or 2) reassign the genetic code to select for which amino acid substitutions are accessible to evolution (Hammerling et al. 2016). This can improve the robustness of a protein sequence's ability to mutate or can enable adaptive mutations, such as Rif^R in generic *E. coli*. Behavior of a non-virulent strain of *E. coli* O157:H7 can be extrapolated to the virulent strain and is an acceptable surrogate for use in field studies (Islam et al., 2004) This was demonstrated after a study by established that Shiga toxin 1 and 2 genes in *E. coli* O157:H7 used in manure or manure slurry had no effect on microbial survival over a 21-month period (Kudva, Blanch, and Hovde, 1998).

Studies, including the study to be mentioned, use Rif^R variants of generic *E. coli* as a way to select and differentiate between inoculated *E. coli* and indigenous *E. coli* during field trials (Lekkas et al. 2016; Gutiérrez-Rodríguez et al., 2012; Sharma and Reynnells, 2016; Moyne et al., 2011; Cutler et al., 2018).

European Food Safety Approach and Policies for STEC in Foods

The EFSA has previously asked the Scientific Panel on Biological Hazards (BIOHAZ) for scientific advice on identification of strains or serotypes of STEC that can cause human illness (EFSA, 2009). In response, the BIOHAZ panel recommended best practices to monitor STEC serogroups and identify them in foods (EFSA, 2007).
In 2009, the EFSA promulgated the "Technical specifications for the monitoring and reporting of verotoxigenic *E. coli* (VTEC) on animals and food (VTEC surveys on

animals and food)" as a way to better monitor and detect verotoxigenic *E. coli* (VTEC) in foodstuffs in accordance to the Directive 2003/99/EC (EC, 2003) (EFSA, 2013). A risk-based sampling approach to monitor for STEC O157 as well as serogroups: STEC O26, O103, O111 and O145 was recommended. The EFSA recommends the standardized ISO 16654:2001 (ISO, 2012; ISO, 2001) methods to detect *E. coli* O157 in food. In 2009, the methods suggested that both *stx* and *eae* genes must be detected using PCR to be considered a positive VTEC isolate and validated by a subsequent test. Meanwhile, in 2013, the European Food Safety Authority (EFSA) (2013) concluded "Strains positive for Shiga-toxin 2 gene (*stx2*)- and *eae* (intimin production)- or [aaiC (secreted protein of EAEC) plus aggR (plasmid-encoded regulator)] genes are associated with higher risk of more severe illness than other virulence gene combinations" (EFSA, 2013). The outbreak of STEC O104:H4 that occurred in 2011 demonstrated the challenges of predicting new STEC types when only screening for the *eae* gene or when focusing on a select few serogroups. Consequently, this molecular approach that characterizes virulence genes other than the *stx* genes is recommended.

The number of samples tested for any given food product will be evaluated based upon the expected prevalence of STEC O157 or other known serogroups to cause human illness based on the number of positives expected to be found in particular food category, which in this case is RTE foods. It requires that such surveillance will occur at a minimum, every three years, although it is suggested that this occurs annually to obtain the number of samples required. Random sampling is suggested from foods, carcasses, or foods from retail outlets. Packaging integrity must be maintained in retail settings, with no signs of damage, to avoid cross-contamination when selecting samples for testing.

Testing for STEC in foods has created a dilemma and food producers are now required to establish their own sampling plan and minimum sampling size (number of samples) (EFSA, 2009) as well as improving preventive controls to mitigate such risks. This is especially relevant for soft and semi-soft cheeses made from raw milk. This allows data to be collected and surveys can identify foods that pose greatest risk for human health based upon probability of STEC occurrence. Although detection of STEC is reported to be low in foods and require time-consuming detection methods, characterization of STEC isolates is key to produce meaningful data for food processors, like cheesemakers, to better understand epidemiological trends. Although a minimum survey of foods is conducted every 3 years, it is suggested that sampling surveys be completed annually to enhance statistical power and see more significant trends. This includes establishing a sample size that includes retail settings. After the first survey at any food production facility, adjustments of sample sizes and procedures used for data collection will be altered accordingly to enhance precision of estimating risk and prevalence of STEC. Guidance also suggests that foods be monitored accordingly and may benefit from survey that are created for the individual producer.

United Kingdom Approach and Policies for STEC in Foods

The UK's Food Standards Agency (FSA) has provided guidance to help food producers comply with good food hygiene standards that are specified in Regulation (EC) No. 852/2004 to avoid cross-contamination with *E. coli* O157 (FSA, 2001). This guidance allows approved organizations of food manufacturers to have established parameters to control cross-contamination. The UK has established testing standards and corrective actions that are associated with STEC detection in RTE cheese products. While, the UK is

aware that Regulation (EC) No. 2073/2005 (as amended) does not specify a criterium for *E. coli* in raw milk cheeses, they require cheesemakers to complete routine testing of final product to monitor and verify that no contamination has occurred. Testing should be completed for products that have previously associated with STEC (VTEC), causing epidemiological concern. The UK Health Protection Agency established "Guidelines for Assessing the Microbiological Safety of Ready-to-Eat Foods Placed on the Market" in 2009 (HPA, 2009). The UK follows a zero-tolerance policy, where in a 25-gram sample of an RTE food, any detection of *E. coli* O157:H7 and other VTEC is considered unsatisfactory as it is deemed "potentially injurious to human health and/or unfit for human consumption". This implies that inadequate or poor processing or cross-contamination have occurred. Suggested corrective actions include further investigation of the food, production processing and environment and further testing of samples and environmental monitoring, with subsequent completion of lab-based confirmatory references tests such as serotyping, phage typing, verocytotoxin typing, and molecular typing (HPA, 2009). ISO/TS 13136:2012 and the draft UK policy specifies that analytic methods for further characterization of STEC must make note of the pathogenic serogroup when one or more *stx* genes are detected, including the *stx* 1 or *stx* 2 virulence markers, and the intimin gene (*eae*) for adhesion (Food Standards Agency and Food Standards Scotland, 2016).

Incidence of Illness and Outbreaks of STEC

More *E. coli* O157:H7 (STEC) outbreaks were reported between the years of 2003 to 2012 in the U.S., when compared to reported outbreaks from 20 years prior (Heiman et al., 2015).

Foodstuffs that are implicated in outbreaks due to STEC contamination likely came into contact with fecal matter. The four primary routes of entry for EHEC include person-to-person transmission, contact with infected animals, transmission through the environment, and foodborne transmission.

STEC was first identified as a pathogen in 1982 when two outbreaks were attributed to consumption of beef patties causing HC in those infected (Riley et al., 1983;Doyle, 1991). *E. coli* O157:H7 has been the leading cause of most outbreaks in North America, Europe and Japan, although other serogroups mentioned are also a public health concern (Farrohk et al. 2013).

The CDC reported 255 outbreaks, 3,667 illnesses, 1,035 hospitalizations and 209 diagnoses of HUS, and 25 deaths related to *E. coli* O157:H7 and foods between the years of 2003-2012 (Heiman et al. 2015). Data collected demonstrated that *E. coli* O157:H7 outbreaks caused 4,928 illnesses, hospitalized 1,272, and led to 33 deaths in the U.S., where dairy products, leafy greens, and fruits were implicated and consisted of 16 (4%), 29 (7%), and 6 (2%) of all outbreaks, resulting in 140, 922, and 57 illnesses, respectively (Heiman et al. 2015). This survey established that 7 deaths were attributed to leafy vegetable consumption and 6 deaths were associated with fruit consumption; no deaths occurred from consumption of dairy products. Of the 16 outbreaks linked to dairy products, 13 (81%) cases were linked to unpasteurized milk and 3 (9%) to cheese made from unpasteurized milk. In the United Kingdom (UK), 1,149 illnesses were reported in 2007 as a result of consuming foods contaminated with STEC. The most recent reports by the European Food Safety Advisory (EFSA) in 2015 stated that STEC caused 5,901 (39.4%) illnesses, 853 (36.3%) hospitalizations, and 8 deaths (0.24%) and were similar to 2014 surveillance data

(EFSA, 2016). In 2015, 2,719 and 1,463 of these cases were associated with dairy products and produce, respectively. STEC outbreaks and illnesses have implicated food commodities including ground meats, unpasteurized and pasteurized milk and milk products, unpasteurized fruit juice, lettuce, spinach, sprouts, and in 2009 was traced to commercially manufactured frozen cookie dough (Little et al., 2008; USFDA, 2012). The EFSA has also reported outbreaks associated with other STEC serogroups. On May 21, 2011, a rare STEC O104:H4 serogroup was isolated from fresh salad vegetables implicated in a multi-national outbreak (Germany, France, and the U.S.) that resulted in 3,911 cases of illness, 850 cases of HUS, and 32 deaths (Muniesa et al., 2012; EFSA 2011). EFSA (2007) reported that between 2002 and 2006, 20% of STEC cases resulting in HUS were associated with non-O157 serogroups; O26, O103, O91, O145, and O111. This is meaningful data as non-O157 serogroups possibly cause 20% to 50% of known STEC infections (Hughes et al., 2006).

Outbreaks Related to Milk and Milk Products

The four primary routes of entry for EHEC include person-to-person transmission, contact with infected animals, transmission through the environment, and foodborne transmission. Following concerns of *E. coli* O157:H7 contamination in foods, STEC was considered an adulterant according to the USDA in 1994, mandating that a step in food processing, such as heat treatment or irradiation, must be implemented to eliminate the pathogen. In 1996, *E. coli* O157:H7 was added to the CDC's Foodborne Disease Active Surveillance Network[12].

[12] https://wwwnc.cdc.gov/eid/article/3/4/97-0428_article

While ground beef is a common vehicle for *E. coli* O157:H7 outbreaks (McDonough et al., 2000), other sources of bovine origin include raw and pasteurized liquid milk and milk products (Gould et al., 2014). A total of 183 foodborne outbreaks reported in the U.S. were attributed to *E. coli* O157:H7 between 1982 and 2002 (Rangel et al., 2005). Of these outbreaks, 5% of *E. coli* O157:H7 outbreaks were associated with raw milk consumption. A domestic outbreak linked to the consumption of cheese took place in Wisconsin in 1998. Vats used to make the raw milk Cheddar cheese were used to make fresh cheese curds. These curds were incorrectly labeled as "pasteurized" and were distributed and sold in six counties within Wisconsin and caused 55 people to become ill (CDC, 2000). During this period of time, raw milk cheeses were the source of six cases of gastroenteritis in England (Strachan, et al., 2005), where one case of HUS in a 12 year old child occurred (CDSC, 1998).

Subsequently, three people from northern England consumed Cotherstone cheese made from unpasteurized cow's milk and were sickened with *E. coli* O157:H7 infections (CDSC, 1999). Environmental samples collected from the dairy herd, slurry, and cheese production were negative for *E. coli* O157:H7 (CDSC, 1999b). In 2003, 13 cases of *E. coli* O157:H7 infection were linked to consuming Gouda cheese products in Alberta Canada and resulted in 2 cases of HUS (Honish et al., 2005). Between 1998 and 2011, 38 and 44 outbreaks were associated with unpasteurized and pasteurized cheeses, respectively, and 9 outbreaks had an unknown pasteurization status. Gould et al (2014) demonstrated that between1998 and 2011, of the 90 outbreaks associated with dairy products, *E. coli* O157:H7 associated with four (11%) outbreaks linked to cheeses made from unpasteurized milk and one (3%) outbreak linked to cheeses made from pasteurized milk.

The most recent STEC outbreak related to dairy products in the U.S. was reported by the CDC, (2011) when. on November 24 of 2010, thirty-eight people were infected with *E. coli* O157:H7 after consuming Bravo Farms Dutch Style Gouda Cheese. The number of individuals who became ill in each state are as follows: Arizona (19), California (3), Colorado (11), New Mexico (3) and Nevada (2). Ages of those infected ranged from 1 to 85 years, with the median being 16 years of age. There have been 15 reported hospitalizations, 1 case of hemolytic uremic syndrome (HUS), and no deaths. Another multi-state outbreak occurred that same year, where raw milk cheeses were implicated with 8 cases of *E. coli* O157:H7 infection, resulting in no HUS cases or deaths (Farrokh et al. 2016).

More recently, in March of 2016, 25 cases of STEC infection were identified in a multi-country outbreak in Europe and resulted in 19 HUS cases (EFSA, 2016). Twelve of these cases were linked to STEC O26 serotype, and another 13 cases tested positive for the STEC O serogroup (i.e. O157) or the *eae* gene, stx1 or stx 2 were detected. The isolates were linked back to a Romanian cheese manufacturer that made cheese from cow's milk that tested positive for *E. coli* 026 that did not have the *stx* genes. Other cheese products that were tested did have the virulence genes. It was suggested that multiple strains were involved in this outbreak from multiple sources according to PFGE analysis.

The review of the literature between 1986 and 2010 specifies that the majority of outbreaks related to cheese products that occurred in the U.S. Europe, and the UK were caused by contamination of soft and semi-soft cheeses that were made from unpasteurized milk. This corroborates the notion that soft and semi-soft cheeses are more susceptible than hard cheeses to surface contamination during ripening (Farrokh et al. 2013). Pasteurized

cheeses were also implicated in outbreaks during this time as a result of post-pasteurization contamination.

Incidence in Fluid Milk and Milk Products

STEC, including *E. coli* O157:H7, are often found in the gastrointestinal tracts of dairy cattle and can readily contaminate raw milk produced on farms (Wells and Shipman, 1991). Yet, incidence of *E. coli* O157:H7 does not show a high prevalence rate in raw milk or in cheeses. Since the early 1990's, *E. coli* O157:H7 is isolated from a very low number of samples; 0 to 16.2% of raw milk samples (Hussein and Sakuma, 2005; Murinda et al. 2002; Cardinal, 1993; Wells and Shipman, 1991). Contamination rates in the U.S. and Canada were 4.2-10% and 2%, respectively 30 years ago (Padhye and Doyle, 1992; D'Auost, 1988). Recent studies reporting raw milk incidence of STEC in U.S. at 3.2% (Cobbold et al., 2008). Water and pests are also vectors that introduce contaminants into the farm environment and are potential intervention sites to mitigate dissemination (Shere, et al., 1998; Wallace, et al., 1997).

Ireland reported incidence in raw milk at 0.8%. While incidence of STEC is low in raw milk samples, virulence genes were detected in 36% and 21% of STEC positive raw milk samples in Ireland and the U.S., respectively. On July 21, 2016, The FDA released a Microbiological Sampling Assignment Summary Report (USFDA, 2016) as part of a preventive sampling approach to eliminate contaminated foods from reaching consumers. Studies have described that the correlation between the presence of *E. coli* O157:H7 on a dairy farm and the presence in STEC in raw milk is undefined (Wells and Shipman, 1991). STEC was only isolated from 1 of 23 (4.3%) raw milk samples

obtained from a farm in Wisconsin. STEC has also been isolated from bulk tank milk of cull cows (0.75%) (Murinda et al., 2002), in South Dakota and western Minnesota (3.8%) (Jayarao and Henning, 2001) and in Pennsylvania (2.4%) (Jayarao et al., 2006) however none of them were of serotype O157:H7. Most recently, 60 *E. coli* isolates were detected from raw milk samples taken from 86 cows with confirmed mastitis, 13(21.6%) of the *E. coli* isolates were STEC (Tavakoli and Pourtaghi 2017). Four (30.8%) of these STEC isolates carried the *eaeA* gene, 7 (53.8%) STEC isolates carried the *stx1* and *eaeA* genes, and only one (7.7%) STEC isolate carried both, *stx1* and *stx2* genes.

Comparably, 162 STEC isolates (88 persistent STEC isolates shedding for ≥4 months) and 74 sporadic STEC (shedding for ≤2 months)) were isolated from cattle in addition to 16 unknown bovine STEC isolates (Barth et al. 2016) and 27.23% of 268 raw milk samples were positive for *E. coli,* where 20.54%, 15.06%, 15.06% and 49.31% of those *E. coli* strains were STEC O26, STEC O11, EHEC, and AEEC, respectively (Momtaz et al., 2012). All EHEC strains carried *stx1, eaeA*, and *ehly* virulence genes and *stx 1* and *eaeA* was found in 77.7% and 55.55% of AEEC strains, respectively.

The FDA analyzed 1,606 samples of raw milk cheeses that were aged for at least 60 days between 2014 and 2016 The FDA found that only 13 (<1%) of these cheeses tested positive for *Salmonella*, *L. monocytogenes*, *E. coli O157:H7* and Shiga toxin-producing *E. coli* combined. STEC was detected in 11 of the 1,606 (0.68%) samples tested. Characterization identified that only one sample of a hard, raw goat milk cheese had a pathogenic *E. coli* O111:H8 serotype resulting in a 0.06% contamination rate. Between 2004 and 2006, the FDA also analyzed 3,360 domestic and imported cheese samples for EHEC, and only detected presence in 3 (0.09%) samples, which included

imported Mexican-style soft and soft-ripened cheeses (D'Amico and Donnelly, 2011). Mexican fresh cheeses were tested for pathogens and found 54% of those samples (n = 200) to have *Salmonella* spp., followed by 16% of samples tested positive for *E. coli* O157:H7 (Torres-Vitela et al., (2012) . These findings corroborate the notion that soft cheeses, especially illegally produced Mexican-style soft cheeses, are more susceptible to contamination (Farrokh et al., 2013; D'Amico and Donnelly, 2011). The EFSA also uses their approach to determine trends overtime with seasonality changes. In 2009, STEC infections occurred more frequently during the summer and fall, with September having the highest amount of occurrences (EFSA, 2009).

Behavior in Cheese Products

There are many hurdles that STEC must overcome to survive in cheeses, such as pH, temperature, water activity, and salt content (Farrokh et al. 2013). Cheese ecology, derived from raw milk microflora and the addition of starter cultures, could cause an antagonistic environment for STEC and inhibit growth through antimicrobial properties (Dineen et al., 1998). Optimal growth temperatures for *E. coli* are between 10 to 46°C (ICMSF, 1996), however challenge studies have shown that *E. coli* O157:H7 can survive and grow in temperatures as low as 7°C in dairy products (Heuvelink et al., 1998) and can persist in acidic environments such as cheeses. The alternate sigma factor gene, *rpoS*, regulates acid tolerance and provides *E. coli* O157:H7 with the ability to survive in pH levels as low as 2.5 for over 2 hours (Baker et al., 2016; Law, 2000). *E. coli* O157:H7 also has protective rpoS-regulated proteins that against heat and salt conditions as seen in Feta cheese (Hudson et al., 1997; Ramsaran et al., 1998), Colby , Romano (Hudson et al 1997), Camembert

(Ramsaran et al 1988), smear rind (Maher et al., 2001) and Cheddar cheeses (Reitsma and Henning, 1996; Schlesser et al., 2006). *E. coli* O157:H7 populations declined within 30 days of aging Colby and Romano cheeses due to acidic pH levels from active starter cultures, temperature, and salt content (Hudson et al. 1997). Microbial populations on surfaces of aged smear rind cheeses produce antimicrobial substances that decrease the presence of pathogens. As a result of the surface microflora, the pH levels of the rind subsequently start to increase (Maher et al 2001). *E. coli* O157:H7 survives initial processing steps during cheesemaking until heat steps are taken (80°C for 5 minutes) as a way to inactivate pathogens in Mozzarella and cottage cheeses (Spano et al., 2003). However, fat molecules may protect STEC during the thermal inactivation step (Erickson and Doyle, 2007). Raw milk that undergoes heat treatment at 65C for 17.6 seconds is adequate to inactivate *E. coli* O157:H7 (D'Aoust et al 1988). However, cooking of curds at 48C for 20 minutes for a Fontino PDO cheeses was insufficient for STEC inactivation (Bellio et al. 2018). Due to these properties in cheesemaking, *E. coli* O157:H7 was not detected in 50 cheeses tested for the pathogen and another 153 soft and semi-soft cheeses manufactured in Belgium from cow, ewe, and goats' milk (Vivegnis et al., 1999). Ripening and storage usually cause STEC populations to decline, however this inactivation is dependent upon strain and type of cheese (Farrokh et al. 2016). For example, *E. coli* O157:H7 could not be detected in Feta and Teleme after a respective 44 and 36 days of ripening. Yet, D'Amico et al., (2010) detected *E. coli* O157:H7 strains in inoculated (1.3 log CFU/mL) Gouda and Cheddar cheeses for over 270 days when samples were enriched. The scientific literature also reports that non-O157 STEC survived in Camembert types for upwards of 20 days (Montet et al., 2009). STEC survival in cheeses is reliant on the cheese

type (soft, semi-soft, semi-hard, hard) and technologies used during cheese manufacture may only injure STEC, allowing potential stressor responses to become up-regulated (Farrokh et al. 2013). With ripening, physio-chemical properties may change and result in the persistence of STEC and even growth (Bellio et al., 2018), stressing the importance of meeting targets in cheese processing that introduce hurdles that mitigate the risk of STEC survival (Farrokh et al. 2013).

STEC as a Pathogen of Concern for Cheesemakers

Cheesemaking primarily involves the fermentation of lactose, with steps that control cheese composition, followed by a process known as cheese ripening (D'Amico, and Donnelly, 2011). The use of added starter lactic acid bacteria (SLAB) and non-starter lactic acid bacteria (NSLAB) is essential to achieve food safety through the production of lactic acid, which decrease the pH and creates a less favorable environment for pathogens like STEC (Trmčić et al., 2017; Beresford et al. 2001). To achieve food safety, the FDA currently requires that all raw milk cheeses be aged for a minimum of 60 days at temperatures of 1.67 °C (35°F) or undergo pasteurization (high temperature/short time; 72°C ≥15 s or vat pasteurization; 63°C for at least 30 min) (Little et al., 2008; Ryser, 2001) according to 21 CFR 133. Previously, STEC was a major pathogen of concern that prompted FDA to consider a requirement that all cheeses be pasteurized as a way to eliminate biological hazards (D'Amico and Donnelly, 2011). While this has not occurred, the EU does require that all raw milk cheeses have a label specifying, "made with raw milk" for consumer knowledge (Little et al. 2008). Although cheeses are generally microbiologically safe, they can be and still are attributed to foodborne outbreaks and

illnesses (Donnelly, 2013).

STEC in a final RTE cheese product is a potential public health concern as it can be detrimental to many immunocompromised individuals, causing potentially severe symptoms (Farrokh et al. 2013). Understanding the behavior of STEC during the cheesemaking process is essential for cheese makers to control and mitigate its' presence, especially on a farmstead level, and to follow regulatory standards.

Cheesemakers also have the concern of regulatory implications and authoritative responses by the FDA such as a voluntary recall, administrative detention, seizure, injunction, mandatory recall, suspension of registration, and sampling of raw milk if STEC is detected as specified under section 402(a)(1) of the previously implemented 2015 Domestic and Imported Cheese and Cheese Products Compliance program (USFDA, 2015). Also, recalls as a result of STEC contamination in foods are very expensive for food producers (Carter et al., 2016).

Because milk intended for cheesemaking can become contaminated with potentially pathogenic STEC, it is important that environmental management occurs on the farm, as there are many vectors that introduce contaminants into the farm environment and interventions are needed to mitigate dissemination (Farrokh et al., 2013), and in food production. It is possible for STEC to form attachments to surfaces and develop biofilms on equipment and piping, particularly stainless steel, if proper sanitation and hygiene standards are not implemented (Farrokh et al., 2013), especially in the presence of other microbial communities (Marouani-Gadri, Chassaing, and Carpentier, 2009). Hence, the

For RTE foods where the hazard analysis reveals a hazard that may appear in the finished product and that may be introduced by environmental exposure, an

environmental monitoring programs (EMPs) is an important tool to control these hazards. This tool is cited in Food Safety Modernization Act (FSMA) regulation with emphasis on *L. monocytogenes*, which is a pathogen of concern in many RTE foods (U.S. FDA, 2018a; Beno et al., 2016; USFDA, 2015).

Outbreaks Related to Produce

More *E. coli* outbreaks were reported between the years of 2003 to 2012, when compared to reported outbreaks from 20 years prior (Heiman et al., 2015). According to surveillance data collected between 1998-2008, EHEC was attributed to 19.3%- 31.5% of outbreaks associated with leafy greens, followed by fruit 6 (11%) and beef with 5 (0.4%) outbreaks, respectively. The CDC also reported that leafy vegetables were implicated in 29 (7%) outbreaks, and responsible for 922 (16%) illnesses, 321(35%) hospitalizations, 53(6%) diagnoses of HUS, and 7 (0.8) deaths between the years of 2003-2012 (Heiman et al. 2015). Leafy vegetables most commonly associated with *E. coli* O157:H7 contamination were lettuce (22 outbreaks; 76%), romaine (3), iceberg (1), mesclun mix (1), spinach (4 outbreaks; 13%), and other unspecified types of greens (3 outbreaks; 10%). Out of all foods, leafy vegetables caused the greatest number of deaths between 2003 and 2012 demonstrating the severity of *E. coli* O157:H7 associated with produce.

In October of 2006, bagged spinach was the cause of an STEC O157:H7 outbreak reported from 26 states, where 199 people were infected. Among those who were ill, 102 (51%) were hospitalized and 31 (16%) developed HUS. One hundred forty-one (71%) of those infected were female and 22 (11%) were children under 5 years of age. Out of those who developed HUS, 29% were children (<18 years old), 8% were 18 to

59 years old, and 14% were 60 years old or older. This outbreak caused three fatalities. Two of the individuals were elderly women, and the other was a 2-year-old child with HUS. *E. coli* O157 was isolated from 13 packages of spinach supplied by patients residing in 10 states.

A subsequent outbreak was associated with romaine lettuce, where 58 people were infected with the outbreak strain of *E. coli* O157:H7 reported from 9 states: Arizona (1), Arkansas (2), Illinois (9), Indiana (2), Kansas (2), Kentucky(1), Minnesota (2), Missouri (38), and Nebraska (1). Those who became ill ranged in age from 1 to 94 years, with a median age of 28 years. Out of those who were ill 59% were female. Out of the 58 individuals affected, 33 (67%) were hospitalized, 3 developed hemolytic uremic syndrome (HUS), and no fatalities were reported. The other individuals could not be accounted for.

Shortly after, on November 2, 2012, another outbreak occurred where Wegmans voluntarily recalled its 5-ounce and 11-ounce shell packages of Organic Spinach and Spring Mix blend. Thirty-three individuals in 5 states were infected with Shiga toxin-producing *Escherichia coli* O157:H7. Out of these persons, 46% were hospitalized, and two persons developed HUS. Once again, no fatalities were reported.

On November 10, 2013, Glass Onion Catering voluntarily recalled numerous ready-to-eat salads and sandwich wrap products after 33 individuals from four states were infected with STEC O157:H7. Thirty two percent of those who were ill were also hospitalized, where two individuals developed hemolytic uremic syndrome (HUS). No fatalities reported.

Most recently, on February 24, 2016, Minnesota Department of Health issued a press release alerting consumers to avoid consumption of alfalfa sprouts distributed by Jack and the Green Sprouts. Eleven people were infected with *Escherichia coli* O157 (STEC O157) from Minnesota (8) and Wisconsin (3). Two hospitalizations occurred, but no one developed hemolytic uremic syndrome and no fatalities were reported.

Another multi-state outbreak associated with *E. coli* O157:H7 contaminated romaine lettuce was reported on June 28, 2018 by the Public Health Agency of Canada, specifying that the outbreak had ended (CDC, 2018[13]). The United States stated that the source appeared to be leafy green, but no type has been identified, although Canada has targeted romaine lettuce as the source of contamination. What contaminated the lettuce and where continues to be an ongoing investigation. This outbreak resulted in 210 infections from 36 states with five fatalities in Arkansas, California, Minnesota (2), and New York (CDC, 2018). Most *E. coli* O157:H7 outbreaks occur during the summer, where shedding occurs most frequently from cattle, explaining the increase of prevalence in processing plants. However leafy-vegetable associated outbreaks tend to be most frequent in the fall, which could resulting from summer planting, irrigation, soil amendments and fertilizers that have more *E. coli* O157:H7 organisms in the summer (Heiman et al., 2015). Cattle density may also play a role in potential contamination as northern states have lower rates of STEC infection, when compared to southern states, with California counties, such as Salinas Valley, being some of the largest lettuce producers.

[13] https://www.cdc.gov/ecoli/2018/o157h7-04-18/index.html

Behavior Associated with Produce

The *E. coli* O157:H7 strain that was isolated from the 2006 spinach outbreak that caused over 200 illnesses and 3 deaths in the U.S. contained a gene (norV, nitric oxide reductase) and may be correlated with increased in ability to cause HUS, and enhance the strain's virulence in comparison to other strains (Markland et al., 2013).

Other studies have also shown that the expression of virulence factors is differentiated in bovine colonization and human infections (Sharma et al., 2011). Under atmospheric packaging (O_2) conditions, 2% of Romaine lettuce samples inoculated with *E. coli* O157:H7 had increased *stx*$_2$ and intimin (*eae*) gene expression when stored at 4C for 9 days. This may lead to more Shiga toxin production, and greater risk of foodborne intoxication regardless of host colonization (Sharma et al. 2011). These same conditions also caused the intimin *eae* gene to increase by two-fold. This suggests that *E. coli* O157:H7 has greater potential for attachment and colonization on the intestinal wall and the formation of effacement lesions in the host. Similarly, virulence factors encoded by *rfbE* were also upregulated under the same conditions. The *E. coli* O157:H7 mutant that lacked the *rfbE* gene did not persist as long as the wild-type, signifying that *rfbE* may also play a role in attachment and colonization onto host intestinal epithelial cells.

Implications for Food Manufacturers: General Hygiene and Sanitation Standards

The EU provides guidance on corrective actions for each food category (EU, 2005). The European Commission Notice (2016/C 278/01) is used as guidance by both the EFSA and the UK to implement food safety management systems (FSMS) into food businesses through the use of pre-requisite programs and Hazard Analysis and Critical Control Point

(HACCP) principles as required by Regulation (EC) No 852/2004: hygiene of all foodstuffs and Regulation (EC) No 853/2004: Pre-requisite programs (EU, 2004; EU, 2016). This includes implementation of good hygiene practices, manufacturing, practices, and product specifications. While the EFSA and UK policies are different, food producers should continue to implement environmental controls that incorporate monitoring, validating, and verification of pre-requisite and HACCP/Food Safety programs to minimize risk of contamination or cross-contamination on farmsteads and in processing environments. The only way that STEC can be controlled in dairy (and other food) production facilities is to set preventive procedures throughout the food chain (Farrokh et al. 2016). These varied detection methods and requirements also begs the question of the priority to harmonize with other international agencies when testing for STEC serogroups and understanding its pathogenicity in foods and in the environment.

Enterotoxigenic *E. coli* (ETEC)

Enterotoxigenic *E. coli* (ETEC) produces hyper-secretions of enterotoxins ST (heat stable) and LT (heat labile), leading to water and electrolyte loss once the toxins induce fluid secretion from the epithelial cells (FDA, 2012). These enterotoxins are encoded on plasmids and are obtained through horizontal gene transfer. ETEC is known for causing gastroenteritis also known as "traveler's diarrhea". Presence of ETEC is often tested for once generic *E. coli* levels have exceeded microbiological standards and provides incentive to question the hygiene and safety of the food product. ETEC outbreaks are infrequent in the U.S and occur more frequently with individuals who travel to foreign countries. This is often associated with developing countries and areas that struggle to

maintain proper hygiene. Outbreaks also tend to occur during time periods in the year where the climate is warmer and wet. The infective dose ranges between 10^6 to 10^9 ETEC cells in adults. However, ETEC may require a smaller dose to infect children or those who are immunocompromised, leaving these populations to be the most vulnerable. Onset of symptoms usually occurs 26 hours after ingestion, but onset of symptoms can range between 8 to 44 hours.

Virulence

ETEC bacteria are able to adhere to the epithelial cells in the intestines by activating the surface-expressed plasmid-encoded colonization factors (CFs) (Turner et al., 2006). The CFs are what differentiate strains within species. Other virulence factors include surface-exposed adhesins including TibA and Tia, which are expressed after the CFsare recognized. Outer membrane adhesin *tia*, encodes a pathogenicity island that attaches heparin-sulphate proteoglycans on the cell surface of eukaryotes. The glycosylated form of the autotransporter TibA directs the bacteria to bind to receptors on epithelial cells. When glycosylation does not occur, TibA promotes aggregation and formation of biofilms. ETEC also has the ability to invade host cells and survive within the cytoplasm or cellular vacuoles. The outer membrane protein *tia* induces ETEC invasion into epithelial cells, allowing internalization. Based on the virulence factors held, ETEC organisms are capable of producing heat-labile and heat stable toxins after they have been ingested. The LT toxin subunits translocate across the inner membrane using the SecYEG translocon (Sec) before forming an AB_5 structure in the periplasm after type II secretion (T2S) occurs across the outer membrane and binds to the LPS. Further binding

of B subunits to the gangliosides on the host cell allow the A subunit increases to activate cAMP, leading to phosphorylation of CFTR, resulting in increased fluid secretion and malabsorption that causes diarrhea. The sTa pre-pro-peptide is also translocated across the inner membrane through the SecYEG translocon, leading to extracellular cleavage of DsbA. This induces binding of the guanylate cycles C receptor (GC-C), which results in increased fluid loss that is also known as diarrhea from an increase in cGMP, subsequently promoting phosphorylation of CFTR. DSbA also catalyses formation of disulfide bonds after STb is translocated through Sec of the periplasm. This secretion induces the TolC toxin to bind to sulfide on the hose cell resulting in Ca^{2+} secretion. This influx of Ca^{2+} triggers calmodulin-dependent protein kinase II further activating the cystic fibrosis transmembrane conductance regulator chloride channel (CFTR). Increased Ca^{2+} levels could lead to the formation of intestinal secretagogues prostaglandin E2 (PGE_2) and 5-Hydroxytryptamine (5-HT), which results in water and electrolyte transport and loss out of intestinal cells, causing the typical ETEC diarrheal symptoms. The *Escherichia coli* heat stable toxin 1 (EAST 1) translocates across the inner membrane similar to Sec, but specific mechanisms are unknown. In vitro studies show that EAST 1 will interact with guanylate cyclase of host cells, resulting in an increase of fluid secretion. SlyA is also a positive regulator of Hemolysin ClyA that associated with the extracellular outer membrane vesicles. This protein then interacts with cholesterol (Chol) moieties that oligomerize and form pores in lipid bilayers of eukaryotic cell membranes, which induce cytotoxicity.

Most Probable Number (MPN)

The FDA identifies the MPN method as a statistically based, multi-step assay consisting of completed phases (FDA BAM, 2017). Ten-fold serial dilutions of five subsamples (50 grams each) are blended with 450 milliliters of buffer. Samples are transferred to test tubes containing lactose- based broth to confirm the presence or absence of gas and acid production via fermenting lactose after incubation at 35°C ± 0.5°C for 48 ± 3 h. Gas positive tubes have aliquots removed to be sub-cultured into a selective broth for *E. coli*, incubated for 24 ± 2 h at 44.5°C, and examined for gas production. If no gas is produced, the cultures are re-incubated and examined again at 48 ± 2 h. to re-confirm gas production. Once positive gas samples are established, aliquots of those samples are cultured on selective agar and incubated for 18-24 h at 35°C ± 0.5°C to isolate colonies for further confirmation with biochemical tests for the identification of *E. coli*. Identifying any 1 of the 5 colonies as *E. coli* is sufficient to confirm an *E. coli* tube as positive. These results are entered into a statistical table to estimate the number or organism present in the sample.

International Standards (International Commission of Microbiological Specifications for Foods, Codex, European Union)

Although STEC is a public health concern, foods must also be tested for the presence of generic *E. coli* according to EU regulatory standards. However, the methodologies used by the FDA are different from those employed by the EU. The EU uses microbiological standards described under ISO 16649-3, "Microbiology of the food chain — Horizontal method for the enumeration of β-glucuronidase positive *Escherichia*

coli," to test for food products[14]. Methods are separated into three parts. Part 1 consists of a colony-count technique at 44 °C using membranes to determine coliform count and 5-bromo-4-chloro-3-indolyl-β-D-glucuronide, also known as TBX (Tryptone Bile X-Glucuronide) Chromogenic Agar. Part 2 also consists of a colony-count technique at 44 °C using TBX Chromogenic Agar. Lastly, Part 3 uses detection and most probable number technique. TBX differentiates *E. coli* based on the β-glucuronidase activity (Verhaegen et al., 2015). Glycosyl hydrolases are a widespread group of enzymes hydrolyzing the glycoside bond in carbohydrates or its derivatives (Arul et al., 2008). β-glucuronidase (EC 3.2.1.31) is a glycosyl hydrolase and hydrolyses β-glucuronic acid residues of glycosaminoglycans (GAGs). In prokaryotes, the GUS of *Escherichia coli* is a well investigated glycosyl hydrolase. The *E. coli*β-glucuronidase gene (uidA) has been sequenced, and it is known to encode a stable enzyme. This ISO/TS 13136:2012 recommended agar medium contains selective agents inhibiting the growth of Gram-positive organisms (Verhaegen et al., 2015).

The EU and various research groups use direct plating as the primary method for testing food products. It is well recognized that direct plating is a more accurate and rapid testing method for determining *E. coli* levels in foods. The MPN method lacks precision and is often inaccurate (Gronewold and Wolpert, 2008). As one study describes, not only is it difficult to compare results between CFU and MPN, but also that *E. coli* may be out competed by other coliforms when using the MPN method (Trmčić et al., 2016).

Outbreaks Related to Milk and Milk Products

[14] https://www.iso.org/obp/ui/#iso:std:iso:16649:-3:ed-1:v2:en

According to the CDC, between the years 2000 and 2008, 16,000 illnesses and 12 hospitalizations were associated ETEC infection. ETEC outbreaks are most commonly associated with consumption of contaminated food or water (FDA, 2012). ETEC appears to be transmitted through fecal contamination and can often be found in feces of asymptomatic carriers. However, ETEC does not seem to be transmitted by interpersonal contact. One of the first outbreaks of ETEC was in 1975, when 2,000 people were infected due to consuming sewage-contaminated water at a national park. Contaminated water served at restaurants and other catered functions have been implicated in several ETEC outbreaks. Examples of foods that are associated with ETEC outbreaks include turkey, mayonnaise, crabmeat, deli food, salads, and Brie cheeses. Many of these foods became contaminated through inadequate hygiene of food handlers during preparation. More recently, between the years of 1998-2008, the CDC reported that no outbreaks of dairy products were associated with ETEC[15].

Staphylococcus aureus

S. aureus is a non-sporeforming, facultatively anaerobic Gram-positive coccus-shaped bacterium that is catalase and coagulase positive. Cells are arranged singly, paired, or in grape-like clusters (Le Loir et al 2003). Staphylococcal food poisoning occurs not as the result of consuming the organism but from ingestion of any 14 staphylococcal enterotoxins (SE A-N) produced by some strains of *S. aureus* (Loir et al., 2003). Between 30-50% of the population carry *S. aureus* in their nostrils and on skin

[15] https://www.cdc.gov/ecoli/outbreaks.html

and hair and can contaminate food before or after heat treatment during processing and handling (Le Loir et al. 2003). *S. aureus* can grow in many foods across a broad range of water activity (a_w) levels (D'Amico, 2008) Usually, a_w is described as measuring the amount of water that is bound or unavailable to microbial populations. Most bacteria exhibit growth at a_w levels of 0.90-0.99, but *S. aureus* has grown in laboratory media at a_w levels as low as 0.86 (Sperber; 1983; Genigeorgis, 1989). Enterotoxin reduction under various a_w levels differ by toxin type with SEA (Qi and Miller, 2000) and SED (Ewald and Notermans, 1988) produced over the range of a_w that best enables growth of *S. aureus*, while SEB and SEC are more easily impacted by changes in a_w. *S. aureus* also has the ability to grow in acidic environments as low as pH of 4.0 (Smith et al. 1983). Once formed, SE's are resistant to heat treatment and low pH conditions that destroy the organism that otherwise produce the toxin (Le Loir et al, 2003).

Virulence

Staphylococcal food poisoning, also known as staphyloenterotoxicosis or staphyloenterotoxemia, is the name of the condition caused by the ingestion of SEs. Symptoms include nausea, vomiting, retching, diarrhea, and abdominal cramps, which develop within 1-6 hours after ingestion of the contaminated food product (Ryser, 2001). Other symptoms may include headache, cold sweats, rapid pulse, transient changes in blood pressure, prostration, and dehydration depending on the individual's susceptibility to toxin and the amount of toxin ingested. Normally, recovery takes 1 to 2 days and rarely leads to hospitalization (Ryser, 2001; FDA/CFSAN, 2008; FDA, 2012).

Outbreaks Related to Milk and Milk Products

Staphylococcal outbreaks have been linked to milk and milk products for over a century with *S. aureus* emerging as a major milk-borne pathogen by the 1930's (Ryser, 2001). However, the proportion of dairy related illnesses from staphylococcal poisoning in the U.S. has declined substantially within the last 40 years due to increased mastitis monitoring, improved hygienic practices, and the use of pasteurization (Ryser, 2001). Although the U.S has seen a decline, *S. aureus* continues to be implicated in foodborne outbreaks related to dairy products in France (De Buyser et al., 2001), particularly cheese and cheese products. Raw milk cheese and fluid raw milk outbreaks are associated with dairy cows with mastitis, and post-pasteurization contamination when handled improperly and transmitted through humans (Ryser, 2001). Production of a heat-stable staphylococcal enterotoxin (SE) prior to pasteurization or as a post-pasteurization contaminant is the cause of illness in *S. aureus* associated foodborne outbreaks.

Some well-known outbreaks in the U.S. associated with staphylococcal foodborne illness include an outbreak that caused 16 individuals to become ill after consuming a pasteurized milk cheese (Altekruse et al., 1998). Milk intended for cheesemaking was contaminated post-pasteurization before any starter cultures were added, enabling the bacteria to grow to levels where SE production occurred (Le Loir et al. 2003). Subsequently, 860 individuals were sickened after consuming contaminated pasteurized milk in 1985. Improper storage allowed *S. aureus* to multiply and produce SE (Evenson et al., 1988). The pasteurization process was unable to eliminate the heat stable toxin, only the viable bacterial cells (Le Loir et al. 2003). Generally, *S. aureus* outbreaks are rather rare because starter cultures outcompete the pathogen, unless contaminated post-pasteurization. Outbreaks associated with Cheddar, Monterey, and Kuminost cheeses

made from pasteurized milk infected 42 individuals as a result of post-pasteurization contamination and inadequate acid development from delayed starter activity (Ryser, 2001; (Zehren, 1968a, 1968b). Raw milk intended for cheese making has also been associated with an outbreak linked to the consumption of raw sheep's milk cheese manufactured in Scotland, where 28 illnesses occurred between December 1984 and January 1985 due to toxin production even though the *S. aureus* organism could not be detected. It was determined that the sheep were infected and carrying the *S. aureus* organism. Notably, *S. aureus* was present in bulk tank milk for two years prior to the first known clinical illness (Bone et al., 1989). *S. aureus* has also been implicated in outbreaks in Brazil in 1987 and 1993 and blamed on contaminated Minas-type cheeses with upwards of 7-8 log CFU/ml that produced enterotoxins A, B,C,D, and E (Sabioni et al., 1988); Pereira et al., 1996). According to the CDC, *S. aureus* was the causative agent for three (10%) of 73 total outbreaks associated with non-pasteurized dairy products between 1993-2006. According to the CDC Multistate Outbreak database, *S. aureus* has not been implicated in any outbreaks related to dairy products in the last 10 years.

Incidence in Milk

Mastitis is commonly caused by *S. aureus* and this organism often infects dairy cows, resulting in outbreaks related to contaminated cheese products that undergo inadequate pasteurization or starter culture activity. Previous research reported *S. aureus* in 25.1% of raw cow's milk (De Reu et al., 2007), 31.7-38% of goat's milk (Jørgensen et al. 2005), and 33.3% of sheep's milk (Jørgensen et al. 2005). Prevalence rates are upwards of 75% and 96.2% of bulk milk tank samples from cows and goats, respectively (Jørgensen

et al. 2005). More current studies have shown that *S. aureus* was present in 63% of milk samples tested from bulk tanks in Minnesota, with 1.3% of those being multidrug-resistant *S. aureus* (MRSA) (Haran et al., 2012). *S. aureus* was also present in 74% of milk samples tested from Canadian dairy farms (Olde Riekerink et al., 2010).

Behavior and Enterotoxin Production in Cheese and Cheese Products

S. aureus behavior and enterotoxin production in cheeses depends on the cheese composition, starter activity and type (Meyrand et al., 1998), acidic byproducts, pH, and competition for nutrients (Genigeorgis, 1989). In the absence of starter culture, *S. aureus* may exhibit growth as the cheese is manufactured into young cheese until other microflora in raw milk outcompete the pathogen (i.e., Tenerife) (Zárate et al., 1997). This may provide an explanation as to why *S. aureus* cannot grow of the surfaces of cheeses as a post-process contaminant (Glass et al., 1995). *S. aureus* growth tends to be suppressed in soft cheeses with added starter culture that results in the production of lactic acid and curd coagulation (Hamama et al., 2002). The best way to mitigate the risk of toxin production (SEC) is to limit cell concentrations to 3 log CFU/ml in raw milk and pasteurized cheeses (Hamama et al. 2002). Previous challenge studies document the decline in populations of *S. aureus* during the draining, salting and ripening processes of goats' milk cheese. A small decline of SEA and a decline in *S. aureus* levels were observed when compared to initial levels of 5-6 log CFU/ml due to the salt content and low pH (Vernozy-Rozand et al., 1998). Because acidic pH levels inactivate *S. aureus* cells, it is expected that *S. aureus* can better persist when Camembert-type cheeses are manufactured due a more preferred environment with an increased pH. Initial inoculum

concentrations of 6 log CFU/g of cheese produced a detectable level of SEA, while inoculum levels of 3 log CFU/ml could not (Meyrand et al. 1998). *S. aureus* also has demonstrated its ability to multiply in hard cheeses such as Manchego (Gómez-Lucía et al., 1992) where SEA and SED is produced when made from milk with levels greater than 4 $\log_{10}$ CFU/ml. *S. aureus* was able to survive throughout the manufacturing and ripening processes as a result of inactive starters resulting in favorable pH levels. It is suggested that heat treatment (80-85°C) during manufacture will help eliminate the *S. aureus* organism and prevent SE production (Glass et al. 1998). *S. aureus* behavior was also observed during the manufacture of three variations of uncooked semi-hard, raw milk cheeses (Delbes et al., 2006). *S. aureus* grew rapidly in the first 6 hours and, then, began to decrease by less than 0.5 log CFU/ml between 6 and 24h, where pH levels influenced growth. Initial milk counts were between undetectable (<10 CFU/ml) and 3.03 log CFU/ml. Within 1 day, *S. aureus* levels reached 2.82 to 6.84 log CFU/g. Enterotoxins were detected in two of the three cheeses, where pH and *S. aureus* levels were 6.6 and 6.5 and 5.06 CFU/g and 5.55 log CFU/g, respectively.

Overall, low levels of *S. aureus* (240 CFU/ml) do not pose a food safety risk, as it requires populations exceeding 5 log CFU/ml to produce heat stable SEs (Jorgensen et al. 2005). Based on this logic, the EU follows a sampling plan with an upper limit of 100,000 CFU/g to determine safety risk of raw milk cheeses (EU, 2005). Initial milk levels seem to be the best prediction model for potential levels achieved in finished cheeses and it is advised that initial milk concentrations be kept below 100 CFU/ml to eliminate any potential SE production in addition to the use of starter cultures to control any subsequent growth (Delbes et al., 2006).

Salmonella spp.

Genus *Salmonella*

*Salmonella*e are straight, usually motile (with the presence of peritrichous flagella), facultative anaerobic, Gram-negative rods (0.7-1.5 x 2-5μm) that are part of the *Enterobacteriaceae f*amily (Holt et al., 1994). According to published classification guidelines, two species are categorized under the genus *Salmonella*: *S. enterica* (synonym: *S. cholerasuis*) and *S. bongori* (synonyms: *S. enterica* subsp. *bongori*, and *S. choleraesuis* subsp. *bongori*). *S. enterica* is further segmented into six sub species (synonyms): *S. enterica* subsp. *arizonae* (*S. arizonae*, and *S. choleraesuis subsp. arizonae*), *S. enterica* subsp. *diarizonae* (*S. choleraesuis* subsp. *diarizonae*), *S. enterica* subsp. *enterica* (*S. choleraesuis* subsp. *choeraesuis, S. enteritidis, S. paratyphi, S. typhi,* and *S. typhimurium*), *S. enterica* subsp. *houtenae* (*S. choleraesuis* subsp. *houtenae*), *S. enterica* subsp. *indica* (*S. choleraesuis* subsp. *indica*), and *S. enterica* subsp. *salamae* (*S. choleraesuis* subsp. *salamae*; (Tindall et al., 2005). The newest subspecies addition is *S. subterannea*, which was published in 2005 (Agbaje et al., 2011). *Salmonella*e are further classified using the Kauffmann-White's scheme and divided among 2,500 serotypes that are characterized according to the presence or absence of distinctive surface chemical structures such as somatic (O), flagellar (H), and capsular (Vi) antigens (Holt et al. 1994). The majority of serotypes are associated with *S. enterica* spp. *enterica* and identified based upon the geographical location of a particular outbreak (i.e. Montevideo, referred to as S. Montevideo) or their antigens. Optimal growth temperatures of *Salmonella* spp.

are 35 to 37°C, with a minimum of 5.5 to 6.5°C, however conditional by serotype. Foods that are stored below 5°C do not support the growth of *Salmonella* spp., however while the optimal pH for growth is between 6.5 and 7.5, *Salmonella* spp. can persist and grow in acidic environments. Factors to consider when determining the minimum pH where *Salmonella*e can grow include acid type, temperature, available oxygen, growth medium, level of inoculum and serotype (El-Gazzar and Marth, 1992).

Virulence

All *Salmonellae* are pathogenic, ranging from symptoms of milk gastroenteritis to typhoid fever (Ryser, 2001). Many cases of salmonellosis go unreported due to gastroenteritis that is self-limiting. Non-typhoidal salmonellosis onset occurs 12-36 hours after the contaminated food is ingested, where symptoms are typically nausea and vomiting and subside within a few hours (Ryser, 2001). Onset of nausea and vomiting are typically accompanied with chills, fever, abdominal pain, and usually diarrhea (El-Gazzar and Marth, 1992; Ryser, 2001). In severe cases, septicemia occurs after complex gastroenteritis distress, which can be fatal for immunocompromised individuals. Extended periods of septicemia can cause localized infection of tissues and organs, targeting those that are already injured or diseased (Ryser, 2001). The number of cells consumed and host susceptibility determines the length and severity of symptoms and can range between 2-6 days (El-Gazzar and Marth, 1992). Typhoid fever, an enteric fever, is associated with a distinct biochemical serovar found in *S. enterica* ser. Typhimurium.

Onset of Typhoid fever lasts between 8 to 15 days and can persist upwards of 30

to 35 days. Fever can reach 40°C (104-105°F) over a period of three to four days, and sometimes up to a week, however severe cases can result in death (Ryser, 2001). Typhoid fever as a result of *S. enterica* ser. Typhimurium infection has a mortality rate of 10% (FDA, 2012). This can be treated with antibiotics, however *S. enterica* ser. Typhimurium cells will continue to shed in patients' feces for 3 months to upwards of a year after antibiotic treatment with prolonged infection (Ryser, 2001). Arthritis-like symptoms may also occur as a long-term sequelae three to four weeks after the initial onset in 2% of cases (FDA, 2012).

According to the CDC, *S. enterica* ser. Typhimurium and S, enterica ser. Enteritidis were the most commonly implicated in the U.S., being responsible for 27.6% of all salmonellosis cases (CDC, 2012). In the early 1980's, multiple drug resistant (MDR) strains of *S. enterica* ser. Typhimurium definitive type (DT) 104 were identified in wild birds, such as gulls, and accounted for almost 70% of 684 isolates found in 2004 at the National Antimicrobial Resistance Monitoring System (NARMS). These MDR strains displayed resistant to ampicillin, chloramphenicol, streptomycin, sulphonamides and tetracyclines (ACSSuT type; Threlfall, 2000; CDC, 2013). Many strains also show encoded resistance within the chromosome against trimethoprim and ciprofloxacin (Threlfall, 2000). Emerging MDR strains of *Salmonella* such as MDR *S. enterica* ser. Typhimurium DT104, are causing concern as infection often results in increased morbidity and mortality rates (Helms et al. 2002). Subsequently, *S. Newport* strains are becoming resistant to amoxicillin/clavulanic acid, ampicillin, cefoxitin, ceftiofur, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (ACSSuT) as well as other third generation cephalosporins or fluoroquinolones (CDC, 2013).

Outbreaks Related to Milk and Milk Products

According to the CDC, non-typhoidal Salmonellosis caused 1,000,000 illnesses, 19,000 hospitalizations, and 380 deaths annually between the years of 2000 and 2008, a slight decline from Mead et al., (1999) reporting 1,412,498 illnesses and 582 deaths annually, with foodborne disease causing 95% of those illnesses. On the contrary, illnesses and deaths attributed to *S. enterica* serotype Typhi have increased from 824 illnesses (Meat et al.1999) to 1,800 illnesses and 200 hospitalizations annually according to the CDC between 2000 and 2008. Because of this increase in illnesses, *Salmonella* serotype Typhi is quickly becoming a large concern. Until the 1940's, *S. enterica* ser. Typhimurium was the first serovar to impact the U.S. dairy industry, casing 50-80% of foodborne illnesses from consuming milk and milk products (mostly raw) (Bryan, 1983; Ryser, 2001). One example of this was an outbreak that began on March 26, 1945 within one week, 250 cases of salmonellosis occurred in six towns within three counties of western Tennessee (Tucker et al. 1946). All individuals had consumed Colby cheese 24-48 hours prior to onset of symptoms, which included chills, fever, nausea, vomiting, and diarrhea. The source of contamination was a creamery in Illinois where the cheesemaker found a dead mouse in the vat and continued to use that milk for cheese manufacture.

Ryser (2001) discusses how challenge studies completed in 1969 and 1970 show that *S. enterica* ser. Typhimurium can persist in hard Cheddar and Colby cheeses, which led to the current FDA regulations indicating that raw milk cheeses must be aged for at least 60 days at a 1.7°C (35°F) or greater. Since hygiene and sanitation standards have increased, along with the use of pasteurization, outbreaks of typhoid fever have not been documented since the 1950s (Bryan, 1983; Ryser, 2001). Regardless, raw and raw milk

products have been implicated in many outbreaks of non-typhoidal *Salmonella* spp. infections since 1965 (Ryser, 2001; Bryan, 1983). *Salmonella* caused over one fourth (331) of the cases in 46 raw milk outbreaks reported in the U.S. between the years of 1973 and 1992 (Headrick et al. 1998). Subsequently, 16 cases of gastroenteritis attributed to *S. enterica* ser. Typhimurium were mentioned after Grade A raw milk was inadequately pasteurized with no monitoring of time-temperature parameters and consumed at a convent in western Kentucky, finding that the inadequate pasteurization was the cause of the foodborne outbreak (Adams et al. 1984). The following year, pasteurized milk was implicated in over 16,000 cases of confirmed *S. enterica* ser. Typhimurium salmonellosis that were reported in six Midwest states (Lecos, 1986).

Raw milk and milk food products were implicated in a multistate outbreak of *S. enterica* ser. Typhimurium infections that occurred from 2002-2003 in Illinois, Indiana, Ohio, and Tennessee. Although the source of contamination could not be identified, it was suspected that any of the four barn workers who had shown symptoms of *S. enterica* ser. Typhimurium infection may have contaminated the milk during the milking, bottling, or capping processes (Holt et al. 2004).

Cheeses have also been implicated in several *Salmonella* spp. outbreaks since 1976 (Ryser, 2001). During that year, continuous *Salmonella* observation in Colorado identified an outbreak of S. Heidelberg that in total caused 339 case of gastroenteritis that were associated with the consumption of Cheddar cheese made from pasteurized milk (Fontaine et al. 1980). The raw milk intended for cheesemaking was not stored under proper temperatures for 1-3 days previously from pasteurization. There were no known complications with pasteurization capabilities, since no phosphatase testing was

completed. This suggests that good manufacturing practices and sanitation standards were lacking. Contaminated cheeses had approximately <1 bacteria per 100 g of S. Heidelberg, considered a low infectious dose (Ryser,2001). After six years, another outbreak occurred in Canada that was associated with contaminated Cheddar cheese made from milk contaminated with *S.* Muenster due to mastitis infected cows shedding the bacteria (El-Gazzar and Marth, 1992; Ryser, 2001). Subsequently, in 1984, 2,000 confirmed cases of salmonellosis from all four Atlantic Provinces and Ontario, Canada were reported from consuming cheese made with pasteurized or heat-treated milk (66.7$_{o}$ C for 16 sec), creating the largest epidemic in Canadian history. *Salmonella* were detected in cheese samples at <10 organisms per 100 g, mirroring the previous 1976 outbreak levels (D'Aoust, 1985). Contamination resulted from improper pasteurization after an employee shut down the pasteurizer while milk was still moving through the system and into the vat (Johnson et al. 1990). After molecular subtyping, it was determined that there were two sources of contamination. The *S. enterica* ser. Typhimurium phage type under subgroup I was obtained from 10 isolates collected from dairy cows and the raw milk intended for cheesemaking while plasmid sequences of group II and I were found in cheeses that were also isolated from employees working at the dairy and their relatives. Infected employees worked in manufacturing or packaging and had direct physical contact with the cheese suggesting this was another source of contamination (Bezanson et al., 1985). Rare serotypes of *Salmonella enterica* ser. Javiana and *Salmonella enterica* serovar Oranienburb were implicated in a multistate outbreak in 1989 that were linked to contaminated Mozzarella cheese and other shredded cheese products manufactured in Minnesota and Wisconsin. One single facility was

considered the source of contamination and implicated cheeses that were also shredded at this facility (Hedberg et al., 1992). It was concluded that the sources of contamination were likely infected employees or cross-contamination from the manufacturing environment. When Mozzarella cheeses were sent to other facilities to be shredded, those facilities were then contaminated, subsequently cross-contaminating other cheese products. Inspections of the facilities revealed that the processing equipment were not regularly cleaned and sanitized between processing different cheeses. *Salmonella* levels found in contaminated cheeses were 0.36 MPN/10 g and 4.3 MPN/100g. All the aforementioned outbreaks confirm that very low levels of *Salmonella* spp. can cause illness (Hedberg et al. 1992). Later that year, an outbreak of 42 total cases, associated with Irish soft cheeses made from unpasteurized cow's milk, occurred in England and Wales (Maguire et al., 1992). In 1993, a nationwide outbreak of salmonellosis occurred in France that was linked to the consumption of cheese manufactured from unpasteurized goats' milk over a 3-month period, resulting in 273 cases of *S.* paratyphi B, and one death (Rampling, 1996). It was established that one of the 40 suppliers of milk was the source of contamination, but the source within that dairy facility could not be found (Desenclos et al., 1996).

In Ontario in 1994, soft cheeses made from unpasteurized milk were sold at a farmer's market and infected 82 individuals with *S. berta.* It was concluded that cheeses were cross-contaminated from chicken remains that were processed in the same facility after subtyping results were the same throughout isolates between patients, cheeses and chickens (Ellis et al., 1998). In 1997, *S. enterica* ser. Typhimurium infections were associated with the consumption of Morbier, a type of raw milk soft

cheese produced in the Jura district of France (De Valk et al., 2000). One year later, *S. enterica* phage type 8 caused an outbreak in Canada that caused 800 illnesses, mostly in children, after consuming lunch packages that contained contaminated Cheddar cheese made from pasteurized milk (Ahmed et al., 2000; Ratnam et al. 1999). In 2001, this same *S. enterica* phage type 8 strain was also implicated in two other community outbreaks located in southwestern France (Haeghebaert et al., 2003).

In 1999, *S. enterica* serovar Oranienburb contaminated cheese made from cow's milk was implicated in an outbreak of 16 cases that occurred in the Austrian province of Tyrol. According to observers on the alpine farm, chickens often entered the dairy as they were allowed to walk freely around the farm. Further investigation found *S.* ser. Oranienburb in environmental and fecal samples from the chicken coop, confirming that the chickens were the source of contamination (Allerberger et al., 2000). Prior to this outbreak in 1997, Washington State health officials identified trends of increasing salmonellosis cases up to 5-fold, particularly in Hispanic populaces (D'Amico, 2008). A confirmed 54 cases were reported, with ACSSuT type DT104 or DT104b being the majority of isolates found in implicated cheeses produced from the same raw milk. However, two different people in different towns made these cheeses. During that same year, two other outbreaks occurred, totaling 110 confirmed cases of MDR *S. enterica* ser. Typhimurium DT104 and DT104b after the consumption of raw milk Mexican-style cheeses produced in California (Cody et al., 1999). These cheeses were made in homes and sold illegally. Vermont also had experienced a small MDR *Salmonella* spp. related outbreak in 1997 that was associated with raw milk obtained from animals infected with the organism (Marcus et al. 1997). Another outbreak of *S. Newport* in northeastern

Illinois was associated with aged Mexican-style cheeses (Coitia) and raw milk from a small dairy, which infected 85 individuals from early 2006 to 2007 (Austin et al. 2008).

The CDC (Painter et al., 2013) also reported that out of a total of 73 outbreaks associated with non-pasteurized dairy products that occurred between 1993 and 2006, 16 (22%) of these outbreaks were caused by *Salmonella* spp. Out of 30 outbreaks associated with pasteurized dairy products, *Salmonella* spp. infection accounted for 6 (20%) of those outbreaks.

Most recently, Gould and colleagues (2014) reported the incidence of outbreaks attributed to pasteurized and unpasteurized cheeses between the years of 1998 and 2011. Results demonstrated that *Salmonella* spp. were implicated in the most outbreaks during this time (other than Norovirus), with 13 (34%) outbreaks related to cheeses made from unpasteurized milk and 6 (18%) outbreaks related to cheese made from pasteurized milk, out of a total 38 and 44 outbreaks, respectively. Ten outbreaks linked to salmonellosis were attributed to unpasteurized *queso fresco* and other Mexican-style cheese types.

Salmonella outbreaks associated with unpasteurized and pasteurized cheese and cheese products typically are sourced back to non-compliance of hygiene, sanitation, and good manufacturing practices related to the environment and employee handling. Raw milk intended for cheesemaking can also be contaminated from animal environments and fecal matter. This stresses the importance of implementing best practices to avoid cross-contamination and mitigate risk of foodborne illness.

Incidence in Milk and Milk Products

As previously mentioned, dairy herds are infected with *Salmonella* spp. that results in symptomatic or asymptomatic fecal shedding of the organism (Ryser, 2001). Mammalian gastrointestinal tracts are common reservoirs for salmonellosis and can result in fecal contamination (El-Gazzar and Marth, 1992). In some circumstances, healthy animals that have mastitis can cause *Salmonella* spp. contamination of raw milk during milking (Fontaine et al., 1980). In Europe, incidence rates of 0% to 2.9% are associated with raw milk samples that test positive for *Salmonella* spp. (De Reu et al., 2007; Desmasures, 1997; Rea et al., 2018). Canada has a very low incidence, reporting 0.17% of raw milk samples testing positive for *Salmonella* spp., while the U.S. has experienced higher rates of 1.5% to 8.9% throughout various geographical areas (Jayarao et al., 2006; Jayarao and Henning, 2001; McManus and Lanier, 1987; Murinda et al., 2002; Rohrbach et al., 1992; Van Kessel et al., 2004) *Salmonella* spp. were also isolated from 1.5% of milk filters at a New York dairy farm. This was a lesser rate than that observed with *L. monocytogenes* detection. *Salmonella* spp. has also been isolated from goat's milk (Foschino et al., 2002). When comparing fecal isolates from dairy cows, it was noticed that small herds of less than 100 animals had a decreased incidence rate of 0.6% compared to those herds greater than 100 (8.8%) (Wells and Shipman, 1991). Overall, only 5.4% of fecal samples tested positive *Salmonella* spp. from 91 herds of dairy cows from 19 states. This data could suggest that farmsteads with smaller herds would have a decreased incidence of *Salmonella* spp. shedding in feces implying that raw milk samples would also have lower incidence of detection. While these studies have used standard culture-based procedures for detection, real time PCR has been used in a study completed by Karns et al. (2005) who detected a significantly higher amount of positive samples

than mentioned previously in other studies. This is comparable to incidence studies completed by (Torres-Vitela et al., 2012) where 200 samples of Mexican fresh cheeses were tested for pathogens and 54% of those samples were found to have *Salmonella* spp., followed by 20% of samples testing positive for *E. coli* O157:H7, and 18% testing positive for *L. monocytogenes*. Brooks et al., (2012) also completed an assessment of 42 raw milk cheeses and found no *Salmonella* spp.

Behavior in Cheese

Previous studies were conducted to observe the ability for *Salmonella*e to survive in hard cheeses such as Cheddar. Hargrove and colleagues (1969) demonstrated that survival of *Salmonella* spp. ranged from 2 to 9 months depending on variation of pH and the amount of starter used. Cheeses that obtained relatively high pH levels did not have inhibitory effects on *Salmonella*e, while cheese with pH levels of 5.32 to 5.3 did show inactivation (Hargrove et al., 1969). *Salmonella* can survive in cheeses at relatively low pH levels for up to 10 months at 7°C (Park et al., 1970). In one study, the survival of *Salmonella* spp. (*S*. Newport, *S.* Newbrunswick, and *S.* Infantis), was observed in inoculated milk intended for cheesemaking and commenced throughout manufacture of Cheddar cheese and storage at 4.5°C or 10°C. While *Salmonella* was present at large concentrations initially (~5 log CFU/ml), *Salmonella* persisted for up to 9 months (El-Gazzar and Marth, 1992; Ryser, 2001). Other studies have also showed similar results where *Salmonella* persisted for up to 7 to 10 months when stored at 13°C and 7°C, respectively. The pH levels were relatively high, at 5.75 and 5.9 respective to 13°C and 7°C (normal Cheddar has a pH of 5.2-5.3) and is the likely reason for pathogen growth and persistence. These

cheeses also held high levels of moisture of approximately 43%. Goepfert et al. (1968) examined stirred Cheddar cheese inoculated with 1-3 log CFU/ml and reported that the mean pH of the cheese after overnight pressing was 5.1, which as significantly lower than previously mentioned studies. This study also recognized that the number of *Salmonella*e decreased by 4 log when aged for 10-12 weeks at 13°C, and 14 to 16 weeks at 7.5°C after an initial increase in populations during manufacture. These studies are relevant to food safety and provide insight to survival trends, however they do not take into consideration the various adaptive capabilities that *Salmonella* spp. obtain under stressed conditions such as acidity and osmolality that promote hostile environments (Leyer and Johnson, 1992). *S. enterica* ser. Typhimurium cell become acid-adapted when they show resistance from presence of organic acids found in cheeses such as lactic, propionic and acetic acids (D'Amico, 2008). These cells are also more resilient during the fermentation process completed by starter cultures when compared to those that are not adapted. When *S. enterica* ser. Typhimurium is a surface contaminant, the organism undergoes acid-adaption in Cheddar, Swiss, and Mozzarella cheeses when they are held at temperatures of 5°C (Leyer and Johnson, 1992). Adapted cells of *S. enterditis* have also survived in cold temperatures and have thrived in cream cheeses of low and high fat content (Smith-Palmer et al., 2001).

Salmonella counts were detectable following enrichment after 210 days in Gouda cheese made from raw milk, surpassing the 60-day aging rule (D'Amico et al. 2014). This study had specified that the mean pH of cheeses tested at 60 days was 5.47. Acid-adaptive cells also have the ability to survive during milk fermentation by lactic acid bacteria, emphasizing the need for using pH as a protective hurdle (Leyer and Johnson,

1992; Leyer and Johnson, 1993).

The Food Safety Modernization Act (FSMA)

On 4 January 2011, President Obama signed the FDA Food Safety Modernization Act (FSMA) into law and it is expected that the Preventive Controls for Human Food law will be fully implemented by September 17, 2018. This was considered the most sweeping reform of the United States food safety laws in more than 70 years by the Food and Drug Administration, introducing changes to the food safety procedures established by the Federal Food, Drug, and Cosmetic Act implemented in 1938 (Trmčič et al., 2017).

FSMA also implements minimum standards for the safe production and harvest of produce based on naturally occurring hazards. This takes biological soil amendments, hygiene, packaging, temperatures, animals in the immediate area, and water quality into consideration.

Through FSMA, FDA will access the records from food facilities to assess for the implementation of food safety systems conducive to manufacturing safe foods. An expectation from FSMA is that food processors will develop and implement improved tracing capabilities on domestic and imported foods. Through the Foreign Supplier Verification Program, importers will have to verify the documentation from foreign facility to ensure foods have been manufactured following US food safety standards (FDA, 2013).

In 2017, the U.S. House of Representatives passed the H.R. 5, the Regulatory Accountability Act (RAA), which would create a more transparent process for

rulemaking and hold federal agencies more accountable (Congress, 2018). The primary goal of the RAA is to ensure that agencies provide the public with the cost associated with regulations before the rulemaking process is initiated and to allow the public to challenge any incorrect or misleading data. The bill entails consideration of (i) the legal authority under which a rule may be proposed; (ii) the specific nature and significance of the problem the agency may address with a rule; (iii) whether existing rules have created or contributed to the problem the agency may address with a rule and whether such rules may be amended or rescinded; (iv) any reasonable alternatives for a new rule; and (v) the potential costs and benefits associated with potential alternative rules, including impacts on low-income populations.

United States Regulatory Policy Regarding Cheese and Cheese Products

The art of cheesemaking and the final product stems from milk quality in terms of both chemical composition and microbiological populations (Johnson, 2013). Traditional cheeses originate from complex systems that have specific sensory characteristics that are linked to factors of biodiversity such as animal feed, indigenous microflora of raw milk, cheese technology and the ripening conditions (Beuvier and Duboz, 2013). Cheeses that originate from Europe, particularly in France, have traditional cheesemaking practices that are protected by geographic indications, which include protected designation of origin (PDO) or AOC (appellation d'origine contrôlée) status. This is vital for the farming and food industry in specific regions. Such examples of cheeses that obtain this AOC or PDO status are made in the Alps or Jura mountains, such as Cote, Beaufort, Abondance, and Emmental, which are all made from raw milk. Over the past two

decades, the U.S. artisanal cheese industry has grown significantly as traditional practices and AOC/PDO status has piqued the interest of consumers (Donnelly, 2013). Artisan cheeses are made on a small scale, use milk that is from traditional breeds of cows, and traditional practices such as bandage wrapping. Out of the 1,400 cheese varieties known to the world today are comprised into the following cheese families: fresh, bloomy rind, smear, ripened, hard uncooked, hard cooked, and blue.

The principle of raw milk hard or semi hard cooked cheeses was to have a food that had a long shelf life so that it would last through the winter (Beuvier and Duboz, 2017). This meant that cheeses needed to be low moisture to reduce microbial growth and slow the aging process to avoid spoilage.

Raw milk cheeses also have a diverse microflora in comparison to pasteurized milk cheeses (Callon et al. 2005). This is known to be the major factor that is responsible for the various flavors and aromas that accompany raw milk cheeses and is very distinguishable by cheese type. Callon et al. (2005) observed the differences in sensory characteristics when three different microbial communities taken from different microfiltered milk filtrates were reinoculated into pasteurized milk during the autumn and winter months. Cheeses made from each of the three different microbial communities at different time points all had different organoleptic specificities. This suggests that the influence of raw milk microflora is independent of when cheeses are made.

While cheeses are generally microbiologically safe, they still cause foodborne outbreaks and illnesses (Donnelly, 2013). Today, much of cheesemaking is based on an industrial scale, where standardization is completed as a result of using specific microorganisms as starter cultures and controlled production.

Cheesemaking primarily involves the fermentation of lactose, with steps that control cheese composition, followed by the cheese ripening process (D'Amico and Donnelly, 2010). Starter lactic acid bacteria (SLAB) and non-starter lactic acid bacteria (NSLAB) are essential in the fermentation process to ensure consistency of cheeses made (Donnelly, 2013). These cultures are also essential to achieve food safety through the production of lactic acid, which decreases the pH and creates a less favorable environment for pathogens (Trmčić et al., 2017; Beresford et al. 2001). The balance of using SLAB, is key to allow acidification of the cheese to inhibit the growth of pathogens, particularly *Listeria monocytogenes,* which is most commonly found soft, soft-ripened and wash-rinded cheeses (D'Amico and Donnelly, 2011; Gould et al., 2014; Kindstedt, 2013). Therefore, regulatory standards established by the FDA state that certain raw milk cheeses that are aged for a minimum of 60 days at temperatures of 1.67 °C (35°F) or undergo pasteurization can achieve food safety (Ryser et al. 2001) according to 21 CFR 133.

While undesirable flavors or physical characteristics are known sensory and quality defects to cheesemakers, growth of undesirable microorganisms or limited growth of desirable organisms is also detrimental. The balance of using SLAB, is key to allow acidification of the cheese to inhibit the growth of pathogens, particularly *Listeria monocytogenes,* which is most commonly found soft, soft-ripened and wash-rinded cheeses (D'Amico and Donnelly, 2011; Gould et al., 2014; Kindstedt, 2013).

Since the early 1900's, raw milk was a primary source of human illnesses, such as tuberculosis and scarlet fever, and led to many deaths that were linked to raw milk (Donnelly, 2013). Pasteurization has been the single most effective intervention to protect public health from consumption of contaminated raw milk. Pasteurization of

fluid milk intended for cheesemaking also ensured consistency and quality of the produced cheeses. Denmark began using pasteurization between 1908-1909 to eliminate any potential pathogens in hard cheeses. Around that time, research on pasteurization in the cheesemaking process to enhance cheese quality and safety was also being conducted at the University of Wisconsin in the U.S. (Donnelly, 2013). Although the conclusion was that cheeses made from pasteurized milk resulted with a safe product, better yield, uniformity, improved shelf life, and made the manufacturing process less complex, pasteurization was meant to eliminate a microbial population that are not the primary pathogens of concern today.

The U.S. regulatory standards that govern cheeses made from raw, heat-treated, and pasteurized milk were first promulgated in 1949 (U.S FDA, 1950; Donnelly, 2013). According to the US Code of Federal Regulations CFR 21 part 1240 (FDA, 2018), all milk and milk products that enter interstate commerce must either be pasteurized or made from dairy ingredients that are pasteurized. Cheeses that must be pasteurized include Monterey, mozzarella, cottage, and cream cheese. Some products are exempt from this standard as described in in 21 CFR part 133, which discusses alternative procedures to pasteurization for cheese and cheese products based on their standard of identity (SOI). The CFR describes SOI based on make procedures, ingredients that can be used, and final characteristics of the cheese or cheese product, including moisture, pH, protein, and milkfat content. The standard specifies that cheeses such as Cheddar, Colby, semisoft, and soft ripened cheeses can be made from raw milk. The current standard for cheeses made from raw milk stipulates that aging of cheeses must occur for at least 60 days at a temperature equivalent to or greater than 35°F (1.7C) according to U.S. regulations (21

CFR 133.182(a). Meanwhile, cheese types such as Asiago medium, Asiago old, and Parmesan cheese must be aged for longer periods of time despite whether or not they are made from raw milk. Despite such stipulations, raw and pasteurized milk cheese and cheese products continue to be associated with foodborne related outbreaks.

Gould et al., (2014) completed an analysis of cheese-associated outbreaks occurring from 1998 to 2011, where 90 outbreaks attributed to cheese were reported. Thirty-eight (42%) outbreaks were due to raw milk cheeses, 44 (49%) outbreaks were from pasteurized milk cheeses, and the other eight (9%) outbreaks were from cheeses where pasteurization status could not be determined. The most common pathogen-cheese outbreak associations were unpasteurized queso fresco or other Mexican-style cheese and *Salmonella* (10 outbreaks), and queso fresco or other Mexican-style cheese and *Listeria* (6 outbreaks) made from pasteurized milk. Thirty eight percent of outbreaks were imported from Mexico and caused by cheeses made in unpasteurized milk.

The most common contributing factors reported in foodborne disease outbreaks associated with unpasteurized milk cheeses were raw product/ingredient contaminated by pathogens from animal or environment (62%), ingestion of contaminated raw products (42%), and insufficient time and/or temperature during cooking/heat processing or reheating (38%) (Gould et al., 2014). The majority of the contributing factors related to foodborne disease outbreaks associated with pasteurized milk cheeses were due to post-processing contamination. These factors were cross-contamination from raw ingredient of animal origin (15%), inadequate cleaning of processing/preparation equipment/utensil (19%), improper cooling or cold-holding (19%), bare-handed contact by

handler/worker/preparer (35%), handling by an infected person or carrier of pathogen (31%), storage in contaminated environment (23%), glove-handed contact by handler/worker/preparer (19%).

Since the FSMA reform, all qualifying food producers must create and implement a preventive controls food safety plan. The 2012 cheesemaker industry survey stated that more than 82% of all cheesemakers within the U.S. will qualify under exemption from needing a written food safety plan (ACS, 2012; Trmčič et al, 2017). The most common constraint that these small-scale artisanal cheesemakers have is limited access to relevant science-based information that would give standard of identity (SOI) to their product and offer food safety assessments to assist with compliance of the FSMA rules.

Aging of some raw milk cheeses has provide a codified standard for cheeses to comply with current regulatory requirements of scientific validation of process controls that generate data on prevalence, survival, and growth of target pathogens in food products (Trmčič et al., 2017). Among other challenge studies, the FDA and Health Canada (2015) recently conducted a risk assessment on the presence of *Listeria monocytogenes* in soft ripened cheeses as one model that illustrates how to distinguish different practices that similarly reduce food safety risk. Testing raw milk and final cheeses for the presence of *L. monocytogenes.* was identified as an example that may provide an equivalent level of protection as pasteurization. Other risk-assessments also emphasize the quality of the raw milk used for the production of soft ripened cheese, Cheddar, Feta and blue (FSANZ, 2009). While risk assessments and challenge studies focus on specific types of cheeses as paradigms, the question has yet to be answered on whether or not conclusions from these individual assessments can be generalized to other

cheese types and varieties. Trmčič et al. (2012) made a valid point, stating that the 60-day age rule will allow cheeses with different characteristics into commerce. For example, in the U.S., a Brie cheese that has been aged for 60 days with a pH above 7 and moisture of 55% is currently held to the same food safety standard as a 2-year aged Parmigiano Reggiano with a pH of 5.4 and 30% moisture. This is also demonstrated with three outbreaks, two caused by *E. coli* O157:H7 and one caused by *L. monocytogenes*, that involved cheeses made from unpasteurized milk that had been aged for longer than 60 days, suggesting that the aging requirement alone is insufficient to render cheese pathogen free (Gould et al, 2014). In one outbreak, facility records indicated that at least some of the lots of implicated cheese had been packaged and released for sale before completion of the 60-day aging period (McCollum et al., 2012). There is empirical evidence that pathogens such as *Salmonella enterica* serovar Typhimurium, *E. coli* O157:H7, and *L. monocytogenes* can survive in cheese beyond the aging requirement of 60 days (Campbell and Gibbard, 1944; D'Amico et al., 2010; Schlesser et al., 2006) and in outbreak settings. Lower-moisture cheeses that are held for 60 days actually supported the growth of *L. monocytogenes* when introduced as a post-process contaminant regardless of the milk type used for cheesemaking (D'Amico et al., 2008, Donnelly, 2013). In response, the FDA completed a risk assessment that modeled relative risk and proposed alternate aging times other than the 60-day aging stipulation as possible preventive controls. *E. coli* O157:H7 could survive well beyond the 60-day aging rule throughout the cheese manufacture process of Gouda and Cheddar when introduced at low levels in raw milk (D'Amico and Donnelly, 2010). Their results were comparable to others that found *E. coli* O157:H7 could be detected in Cheddar up to 130

days using enrichment methods and up to 158 days when raw milk was inoculated at 1,000 CFU/ml (Reitsma and Henning, 1996). *E. coli* O157:H7 can survive between 210 days to 1 year of aging at inoculum doses ranging from 1 to 100,000 CFU/ml (Schlesser et al., 2006; D'Amico and Donnelly (2010).

While science-based interventions and methods have been developed for cheesemakers to validate the safety of each cheese throughout commencement of cheesemaking, seasonal variations in milk quality, composition, and even aging, make this approach costly and timely for a small or very small business by FSMA definition.

This provides the premise for the categorization scheme suggested by Trmčič et al. (2017) that provides 30 general categories as a tool to systemize the safety risk of cheeses. This was used to (i) assess the risk of survival and growth of *L. monocytogenes.* and other pathogens, and (ii) to continue evaluating the effectiveness of different interventions such as the 60 day aging rule for cheeses that pose high food safety risk make procedures and product characteristics. These categories are based on five ranges of pH and water activity (a_w), as they are characteristics that impact pathogen growth and survival. The categories of pH and a_w levels are (A) <4.60%, (B) 4.61-5.00%, (C) 5.01-5.40%, (D) 5.41-5.80%, (E) 5.81-6.20%, (F) >6.20%, and (1) ≤0.920%, (2) 0.921-0.940%, (3) 0.941-0.960 %, (4) 0.961-0.980%, (5) >0.980%, respectively. Researchers collected 273 cheeses in New York State that were ready for sale and distribution and tested each cheese for pH and a_w. Results showed that the 12.4% of cheeses had a pH level above 6.2 and a_w values of 0.961 to 0.980. In this example, cheeses that most commonly fell in the high pH, high a_w categories were washed rind cheese, mold ripened cheese, and low-acid Hispanic style cheese, which have been notoriously known as high-

risk foods. This characterization of cheeses could be used as a tool to determine appropriate preventive control measures for high and low risk cheeses in a pragmatic fashion. It is also applicable to the monitoring requirements mandated by FSMA (FDA, 2015).

However, this approach does not take into account the cooking step utilized during the production of some raw milk cheeses. Bachmann and Spahr (1995) tested Swiss hard cheeses made from raw milk and did not detect pathogens beyond 1 day. Pathogens in these cheeses, including *Aeromonas hydrophila, Campylobacter jejuni, Escherichia coli, Listeria monocytogenes, Pseudomonas aeruginosa, Salmonella* spp., *Staphylococcus aureus*, and *Yersinia enterocolitica* succumbed to a cooking step in the cheesemaking process at a temperature of 53°F, which would eliminate pathogens. Similar findings were also established for Italian Grana cheeses (Donnelly, 2013). The term "raw milk" cheese is misleading because the cooking step during the making process is not taken into consideration. The Australian Food Safety Authority completed thorough risk assessments and concluded that raw milk Swiss cheese varieties (i.e. Emmental, Gruyere, and Sbrinz) and extra hard grating cheeses, including Parmigiano Reggiano, Grana Pdano, Romano, Asiago, and Montasio that undergo manufacture and aging parameters achieve an equivalent microbiological safety standard as cheeses made from pasteurized milk. Tmrcic et al. (2017) also mentioned that the pH in a soft ripened cheese could vary by more than 3 units between the center and the surface of the cheese. Researchers had chosen worst-case scenario and had used the higher pH in their study for the analysis, however more challenge studies are warranted because the performance of

any particular pathogen as cheese ecology is complex and does not comply to such generalization.

The pathogens of concern in 1949 are not the same pathogens of concern that we are focusing on today (i.e., *L. monocytogenes*, *Salmonella enterica* serovar Typhimurium DT104, and *Staphylococcus aureus*) (Donnelly, 2013). In 1997, the U.S. FDA requested that the National Advisory Committee for the Microbiological Criteria for Foods reassess the 60-day aging rule for raw milk cheese manufacture. During this time, in 1996, Canada proposed an amendment that would require all cheeses to be made from pasteurized milk or an equivalent process. However, this was withdrawn in response to a scientific expert committee arguing that these requirements could not be met due to the technical requirements used by small-scale cheesemakers (Donnelly, 2013).

The FDA requires that all cheesemakers follow the Domestic and Imported Cheese Compliance Program (DICCP) that was initially promulgated in 1998 (D'Amico and Donnelly, 2011) with the intentions to conduct inspection of domestic cheese firms, and to examine samples of imported and domestic cheeses for microbiological contamination, phosphatase and filth, generally recognized by the Federal Register as a result of rodent or insect infestation (USFDA, 2002). Each cheese sample was analyzed for six attributes, which included: 1. *Listeria monocytogenes,* 2. *Salmonella*, 3. *E. coli* and Enterotoxigenic *E. coli* (ETEC), 4. Enterohemmorrahagic *E. coli* (O157:H7), 5. *Staphylococcus aureus* and 6. Phosphatase. ETEC analysis was required only when *E. coli* was present at $\geq$10,000 MPN/g, but all other pathogens were tested directly (D'Amico and Donnelly, 2011). For the pathogens of concern, the compliance program allowed a level of 10,000 MPN/g for

generic *E. coli* as an indicator organism, 10,000 CFU/g for *S. aureus*, and a zero-tolerance policy for *L. monocytogenes, Salmonella*, and *STEC* (*E. coli* O157:H7).

The United States FDA, in conflict with EU and ICMSF (International Commission of Microbiological Specifications for Foods) guidance, issued its 2009 Compliance Policy Guide (CPG) which states "The presence of *Escherichia coli* in a cheese and cheese product made from raw milk at a level of greater than 100 MPN/g indicates insanitary conditions relating to contact with fecal matter, including poor employee hygiene practices, improperly sanitized utensils and equipment, or contaminated raw materials" (U.S. FDA, 2009).

The 2009 CPG document was made available for public comment and FDA received 4 comments, one of which was from the American Dairy Products Institute, which stated "in our view, the permissible level of *Escherichia coli* should be set according to standards of food safety without regard to the treatment of the milk itself. Stated another way, the guidance should be set at a uniform level to ensure food safety across all covered dairy products" (U.S FDA/HHS, 2014).

In response, FDA issued 2010 CPG Guidance, stating that for non-toxigenic *Escherichia coli*, "Dairy products may be considered adulterated within the meaning of section 402(a)(4) of the Act (21 U.S.C. 342(a)(4), in that they have been prepared, packed or held under insanitary conditions whereby they may have become contaminated with levels greater than 10 MPN per gram in two or more subsamples or greater than 100 MPN per gram in one of more subsamples" (U.S. FDA, 2010).

This guidance was subsequently revised, and on July 30, 2015, the FDA reissued the Domestic and Imported Cheese Compliance Program guidelines (U.S. FDA, 2015). In the

new guidance, FDA established another 3-class sampling plan for limits on *E. coli* in domestic and imported cheeses (n = 5, c = 3, m = 10 MPN/g, M = 100 MPN/g). If *E. coli* levels exceed 10 MPN/g but are less than 100 MPN/g in three or more subsamples, or greater than 100 MPN/g in one or more subsamples, the cheese is considered adulterated. According to this guidance, if cheeses exceed the proposed 3-class sampling plan, the FDA has the authority to (i) submit a warning letter, (ii) hold a regulatory meeting with the facility, (iii) seize an adulterated lot, (iv) detain/refuse product, (v) add the firm or product of concern to an import alert 12-10, (vi) increase import sampling of the product, or (vii) consider inspection of a foreign firm.

International Standards

It is notable that the EU did not establish a sampling plan for *E. coli* in raw milk cheese (EU, 2005). *E. coli* does not offer a meaningful hygienic index in raw products as its presence is expected, consistent with guidance from ICMSF. ICMSF outlines end product testing criteria for cheeses in Table 23.7 of Book 8 (ICMSF, 2011). This logic is supported by other studies that have also reported presence of coliforms (>10 CFU/g) in raw milk, as well as determining *E. coli* presence in cheeses. In addition to pasteurization, the cheesemaking procedure and aging process reduces the prevalence of coliforms in raw milk cheeses due to acidification and decline in water activity.

This is consistent with studies showing that the prevalence of coliforms declined in the cheese in comparison to levels found in the raw milk. D'Amico et al. (2010) found that 29.8%, 31.4%, 23.1%, 11.6%, and 4.1% of raw milk samples (n =121) tested from Vermont farms had coliform counts of <1, 1-10, 11-100, 101-1,000, and 1,001-10,000, respectively. Other studies reported that coliforms (>10 CFU/g) were found in

56%, 80%, and 87% of raw milk samples tested (Jayarao and Wang, 1999; Pantoja et al, 2009; Jackson et al., 2012). However, a study conducted by Trmčič et al. (2016) only detected coliforms (>10 CFU/g) in 42% of raw milk cheeses tested (n=185). While pasteurization, pH, water activity, and rind type are all factors that impact presence of coliforms, water activity may be the one factor that consistently determines the concentration at which coliforms are present in cheese (Trmčič et al., 2016).

Trmčič et al. (2016) identified coliforms in cheeses by sequencing part of the 16S rDNA using polymerase chain reaction (PCR) and identifying the genus and species through the Ribosomal Database Project (RDP) classifier. These methods allowed researchers to determine and compare coliform profiles in pasteurized and raw milk cheeses tested. Coliforms *Escherichia*, *Enterobacter,* and *Hafna* were the most common genera found in raw milk, but the least common in pasteurized milk. Not all coliforms are pathogenic. Coliforms such as *Hafnia, Raoultella*, and *Serratia* improve cheese quality. *Hafnia* and *Raoultella* are primarily found on the rinds of cheeses and improve textural and sensory properties by producing proteolytic enzymes that break down casein (Hervert et al, 2016; Trmčič et al., 2016). *Hafnia alvei* is a large contributor to aromatic compounds.

This study also established that positive coliform tests (>10 CFU/g) do not provide any additional information about the presence of *L. monocytogenes*. Out of the 273 cheese samples tested (88 pasteurized cheeses, 185 raw milk cheeses) no statistical association was found between presence of coliforms and *Listeria* spp. in cheese, yet statistical significance was found between *Listeria* spp. and cheeses with washed rinds. Researchers believe that this is likely due to surface pH, rather than water activity. and

The presence of *Listeria* spp. predicts only 4.4-6.7% coliform levels (Trmčič et al., year). Prevalence declined from 1.8-1.3% when comparing presence of *L. monocytogenes* to coliform levels.

While coliforms are thermolabile and do not survive pasteurization; it is possible for pasteurized cheeses to contain coliforms due to post-process contamination. Coliforms (>10 CFU/g) were found in 21-29% of cheeses made from pasteurized milk (Trmčič et al., year). The International Dairy Federation states that pasteurized milk cheeses should be tested for *E. coli* one to two weeks after ripening when *E. coli* levels are at their highest (International Dairy Federation, 2016). Therefore, a sampling plan where n=5, c=3, m=10 and $M=10^2$ had been internationally standardized for the detection of *E. coli* in pasteurized and thermized cheeses.

The United States (U.S.) and the European Union (EU) have yet to agree on one microbiological standard that will promote cheese safety. During 1947, rules were established for free trade by the General Agreement on Tariffs and Trade (GATT) (Evans, 1968; WTO, 1998). Subsequently, countries imposed unwarranted safety requirements as a way to protect small industries. In 1994, as a result of unsatisfactory regulations, the Sanitary and Phytosanitary (SPS) Agreement was implemented to resolve complications with decade old non-tariff barriers (Froman, M., 2014; ITCD, 1999). Once the SPS was created, countries became entitled to preserve the health and life of their consumers, animals and plants against pests by protecting them from diseases and other threats. Consequently, the Appropriate Level of Protection (ALOP) was promulgated to prevent measures that are unjustified from hindering international trade, although the U.S. and Europe have yet to achieve a harmonized ALOP for cheeses.

In 1997, the U.S., France and the International Dairy Federation (IDF) formed the Codex Committee on Food Hygiene to create a draft outline of the international milk code (FAO, 1996) titled, "Proposed Draft Code of Hygienic Practice for the manufacture of un-ripened cheese and ripened soft cheese". The U.S. had initially proposed, "Pasteurization, or an equivalent measure approved by the official agency having jurisdiction, shall be used in order to achieve the ALOP." France disagreed declaring, "common hygiene provisions provide adequate health protection without pasteurization" (U.S. FDA, 1999; ITCD, 1999). Regardless of this pushback, the U.S. continued to state that "raw milk and raw milk products are potentially hazardous foods that support growth of pathogens such as *Listeria*, *Salmonella, E. coli* and others. Cheese poses a particularly high health risk because it is usually ready-to-eat (RTE) and will not be cooked before consumption. Scientifically accepted processes control the threat. These can include pasteurization, heat treatment, sterilization of milk; aging of cheese and new technologies not yet developed." The EU commission had then countered that argument, stating, "Consumer safety is protected when strict veterinary and sanitary practices are followed from production to consumption including: using raw milk from herds in good health with regular veterinary inspections/subject to sanitary controls; using milk that is collected, transported and transformed within a short period of time applying strict hygiene; educating consumers about proper storage/shelf life." (U.S. FDA, 1999; ITCD, 1999).

These changes in the FDA DICCP and further comparison to the EU and international standards and risk assessments have established the background for the study and findings described in Chapter 2 of this dissertation.

Environmental Sampling and the Role of Wooden Surfaces in Cheese Processing Facilities

While cheeses are generally microbiologically safe, they still are attributed to foodborne outbreaks and illnesses (Donnelly, 2013) as *L. monocytogenes* contamination is commonly found in ready-to-eat (RTE) foods (Buchanan et al., 2017). RTE foods, such as cheeses, are consumed raw, or are handled, processed, mixed, or cooked without application of other listericidal processes (Buchanan et al., 2017). High doses of *L. monocytogenes* have been found in some cases where RTE foods have been stored at refrigerated temperatures for long periods of time. Ready to eat (RTE) foods are exposed to environmental *Listeria* spp. from post-pasteurization or post-process contamination prior to packaging (Buchanan et al., 2017). *Listeria* spp. are commonly found in agricultural and food processing environments due to the traffic of employees or equipment, improper cleaning and sanitizing, or due to poor equipment design, where certain strains are continuously found and isolated from processing environments (Borucki et al., 2004; Ho et al., 2007; Wulff et al., 2006). Since the 1990's, improved control measures have decreased the prevalence of *L. monocytogenes* in several food categories, especially meat and meat products (Buchanan et al., 2017).

This regular isolation of *L. monocytogenes* from processing environments includes cheese manufacture and addresses the need for artisanal cheesemakers to implement environmental monitoring programs (D'Amico and Donnelly, 2009). *Listeria* spp., particularly persistent strains, continue to contaminate and colonize within food processing facilities (Ferreira et al., 2014; Ho, Lappi and Wiedmann, 2007; Borucki et al., 2004). This

causes concern as it is becoming clear that *L. monocytogenes* is becoming difficult to eradicate from environmental surfaces due to its enhanced fitness and possible resistance to sanitizers (Poimenidou et al., 2016). RTE foods are a major route of transmission for foodborne listeriosis; responsible for approximately 99% in the cases in the U.S. (Buchanan et al., 2017; Scallan et al., 2011).

Wooden materials were used during cheesemaking to contribute to the organoleptic quality of some products. Wood is also lightweight, mechanically resistant to shock, and can sustain integrity in high moisture environments (R. Ismaïl et al., 2015). For example, wooden shelving is seen as an essential part of the manufacture and cave aging process for Roquefort cheeses according to the Food Standards Australia and New Zealand (FSANZ) (FSANZ, 2005). FSANZ completed comprehensive risk assessment similar to the *Codex Alimentarius* guidance that provides risk-based assessed microbiological criteria for international commerce. FSANZ determined that Roquefort manufacturers implement controls to ensure food safety, although these practices are not standard food production conditions. The use of wooden vats when making cheeses such as Ragusano or Pecorino Siciliano is also a required and a PDO protected practice in Sicily (Cruciata et al., 2018).

Although some of these wooden tools have been replaced with food grade plastic or stainless steel materials, wood is still a primary material used for aging shelves during cheese affinage or as a packaging material (Aviat et al., 2016). Approximately 500,000 tons of cheeses are aged on wood shelves annually (Lortal et al., 2014), which many of these cheeses are included under AOC and PDO French cheese varieties, such as Comté, Reblochon, Beaufort, Munster, Cantal, and Roquefort (Donnelly, 2015).

Environmental Monitoring Program Regulations and Guidance

Environmental testing is necessary to detect microbiological niches in the processing environment. The food industry has developed several protocols to locate the areas harboring microbial pathogens and remove them. These protocols describe "seek and destroy" practices to reduce the appearance of niches that serve as potential sources of contamination (Malley et al., 2015).. In the case of controlling *Listeria monocytogenes* in the environment, the stringency of sanitation is of extremely important and these protocols resort to the use of different tools, including tools for verification of sanitation programs, which become the most important program in the control of pathogens in food processing environments.

Environmental testing is necessary to detect microbiological niches in the processing environment, which is defined as "locations harboring the organism after the routine sanitation process for that area has been completed" (Ferreira et al., 2014). Tompkin, (2002) states that a niche is a problem in food production facilities because they are difficult to clean and sanitize and could lead to persisting strains of *Listeria* becoming part of the usual microflora within the environment when sanitation of these areas does not eliminate the organism (Norton et al. 2001b).

Many RTE foods are introduced to pathogens once cross-contamination from an environmental surface occurs, increasing the risk foodborne outbreaks and human illness (Ismail et al., 2017). Recontamination of RTE foods is often a result of inadequate storage parameters or poor storage conditions.

Food contact surfaces need to be properly cleaned and sanitized to reduce cross-contamination. Moisture, amount of contact, and pressure may lead to higher transfer from

a given surface to a food product (Perez-Rodriguez et al. 2008). The type of surface material also plays a role bacterial adhesion, which impacts bacterial transfer (Lahou and Uyttendaele, 2014). While less porous stainless steel and plastic materials have been increasing in popularity and incorporated into food production facilities, wood materials have never been associated with an outbreak, and wood has been found to be just as hygienic as the other food contact materials (Aviat et al., 2016). Additionally, wood provides potential antimicrobial properties in addition to a beneficial microbial ecology that contributes to healthy affinage parameters such as acid production and microbial variety that promote protective properties for cheeses (Di Grigoli et al., 2015).

Controls during production may help minimize risk of contamination of soft cheese made from raw or pasteurized milk to avoid subsequent concerns to public health (McLauchlin et al. 1990). Wooden materials commonly used in the artisanal cheese processing environment and their porosity has led to regulatory concerns, such as shelves used for cheese aging (Donnelly, 2015). The FDA had previously stated "The use of wooden shelves, rough or otherwise, for cheese ripening does not conform to cGMP requirements, which require that "all plant equipment and utensils shall be so designed and of such material and workmanship as to be adequately cleanable, and shall be properly maintained", in 21 CFR 110.40(a) (Donnelly, 2015). After uncertainty from the public, questioning the lack of empirical support when these regulations were proposed, the FDA had retracted this statement, allowing wooden boards to be used during aging of cheeses according to their Center for Food Safety and Applied Nutrition (CFSAN) constituent update on June 11, 2014. Interestingly, the FDA original proposal had overlooked the cited researchers' suggestions of heating wooden boards to 176^{o}F for 5 minutes or 149^{o}F for 15

minutes after cleaning as a way to control pathogens (Zangerl et al., 2010). Additionally, the Food Safety Modernization Act (FSMA) promulgated regulatory standards that require all food producers to implement preventive controls based on the potential hazards of the food product that incorporate monitoring to ensure that preventive controls are being implemented successfully and effectively (Tmrcic, 2017). The FDA's proposed guidance ignores that artisanal cheesemakers are monitoring all environmental surfaces, including wooden materials, for *Listeria* in order to comply with FSMA Proposed Rule for Preventive Controls for Human Food (Donnelly 2015).

According to European Parliament, wooden materials used during cheesemaking are considered an acceptable food contact material according to the Regulation (EC) No 1935/2004 and 852/2004 and by the Council on October 27, 2004, establishing that wood is a material that may come into contact with food as stated in Directives 80/590/EEC and 89/09/EEC (Aviat et al., 2016). The French arête also identified wooden materials as acceptable for food contact in November of 1945. The European commission regulation (EC) no. 2074/2005 allows exemption from (EC) no. 852/2004 for foods such as PDO cheeses made in Sicily and France, which follow traditional practices "as regards the type of materials of which the instruments and the equipment used specifically for the preparation, packaging, and wrapping of these products are made" (EU, 2074/2005). The food safety guidelines established by the European Union (EU, 2074/2005; Melo, Andrew, and Faleiro, 2015) states that throughout the shelf life of a RTE food, *L. monocytogenes* should not grow beyond 100 CFU/g. Similar standards are suggested in the international *Codex Alimentarius* microbiological guidelines for controlling *L. monocytogenes* in ready to eat foods (Melo et al., 2015; CAC, 2007).

While wood has been safely used during cheese manufacture for centuries, studies that were conducted in the 1990's have caused concern for the use of wooden materials, such as shelving, during cheese manufacture due to its porous nature and the potential for the formation of biofilms when compared to other alternative materials, making it difficult to disinfect (Aviat et al., 2016). Hence, environmental surfaces that incorporate sanitary, non-porous design that can be easily cleaned and sanitized (Kabuki et al., 2004), such as stainless steel and food grade plastics, are replacing wooden materials (Ismail et al., 2017). These materials are viewed as superior due to durability, resistance to corrosion, and longevity, decreasing the likelihood of impact damage, resulting in improved hygienic properties during their time of use (Kusumaningrum et al., 2002). Since then, studies have been focused on the efficacy of cleaning and sanitizing wooden boards, as well implications of moisture content and wood type.

Colonization and Persistence of Microorganisms on Surfaces

Biofilms consist of extracellular polymeric materials and many cells that protect individual cells from stressors and support interactions between organisms for nutrients, metabolites, and genetic material (i.e., horizontal gene transfer) that promote survival and growth (Ferreira et al., 2014). According to Kadam et al. (2013), 143 strains of *L. monocytogenes* are capable of forming biofilms, with variation on time and temperature parameters and media or foodstuffs used. Furthermore, the majority of 32 strains found in produce and dairy processing environments can form biofilms on stainless steel and glass (Bonsaglia et al. , 2014). Bacteria in biofilm may develop resistance to antibiotics and even sanitizing agents. In addition, bacteria in biofilm may became persister cells and

serve as the source of contamination for foods (Buchanan et al., 2017). There is a need to advance our knowledge of biofilm detection, removal and prevention in food processing environments to reduce potential sources of cross contamination.

Wood is naturally inhabited with microbes due to the moisture content, rate of decay, the length of time it is stored after it is removed from a tree, and if any additional water is introduced to the material after it has been displaced (Avait et al., 2016). Microbial populations identified are not food-borne pathogens but common microorganisms that are found in the soils or roots of plants and their rhizospheres. However, there is empirical evidence that the microbes on wood and plastic surfaces do not correlated with the surface of cutting board, regardless of food grades (Abdul-Mutalib et al., 2015). A plethora of 40 bacteria of food-borne and otherwise indigenous microbial populations were identified using Polymerase Chain Reaction (PCR) and pyrosequencing methodology. Microbial communities were similar regardless of the food premise location (Avait et al., 2016).

However, the ability for *L. monocytogenes* to harbor and colonize in processing plants is an ongoing issue and is commonly due to inadequate cleaning and sanitizing, poor design of equipment or plant layout, and insufficient controls in the processing environment (Buchanan et al., 2017). These biofilms can be formed on food contact surfaces (FCS) and (NFCS) within production facilities such as cutting boards, conveyer belts, stainless steel equipment, drains, ventilation, floors, and refrigerated storage areas (Lahou and Uyttendaele, 2014). Once *L. monocytogenes* colonizes food-processing plants, etiological and physiological traits allow the organism to persist in food products that are stored at low temperatures (Buchanan et al., 2017).

Kyoui et al. (2016) demonstrated that biofilms were more resistant to sodium hypochlorite when higher levels of glucose concentrations were available (1.0 or 2.0%) when compared to those formed at low glucose concentrations (0.1%). This knowledge can be used to help design and implement appropriate sanitation strategies as these residual food stuffs can support biofilms and become vectors for cross-contamination of food contact surfaces, subsequently contaminating other food products (Ismail et al., 2017; Melo et al., 2015). Bonsaglia et al. (2014) also determined performance of 32 strains found in produce and dairy processing environments and found that the majority of these strains could form biofilms on stainless steel and glass with variation from time and surface type. *Listeria* adhesion is usually based upon the ability to attach to surface and form biofilms as a way to survive during stressors that vary overtime such as temperature, pH (<4.4), and water activity (<0.94) (Kathariou, 2002; Ortiz et al., 2010).

Surface type significantly impacts the ability of microbes, such as *L. monocytogenes,* to attach (Lahou and Uyttendaele, 2014) despite reports that surface roughness and finishes of stainless steel do not affect the recovery of *L. monocytogenes* (Rodriguez, Autio, and Mclandsborough, 2008). Wet surfaces yield a better recovery rate than dry surfaces and may be attributed to inactivated cells when the environment is low in moisture and nutrients are limited (Lahou and Uyttendaele, 2014). Alternatively, *L. monocytogenes* may attach better to surfaces after drying on different environmental materials (Norwood and AGilmour, 2001). In that case, specific cellular structures (such as flagella, pili, and other extracellular polysaccharides) may affect bacteria adhesion and survival under static conditions (Poimenidou et al., 2009). On the contrary, flagellum-mediate motility may influence adhesion and biofilm formation (Lemon et al., 2007). This

discrepancy could be due to variation in pH, oxygen tension, and nutrient availability between studies (Poimenidou et al., 2009). All of these factors may influence how well an environmental food contact surface, such as wood, may harbor potential pathogens.

Studies have been done to specifically determine how well bacteria, specifically pathogens of concern, can harbor within the porous wood material (Avait et al., 2016). Standard swabbing and culturing methods are not as effective at recovering pathogens from wooden surfaces, even when using stomaching or brushing techniques as a means to invade the porous material (Rached Ismaïl et al., 2013). However, grinding was a reliable method for pathogen detection from various wood types, including popular, pine, and spruce (Ismaïl et al., 2013). On average, 30.1% for both *L. monocytogenes* and *E. coli* were detected on spruce and poplar, respectively, and 30.4% recovery for *Penicillium expasum* on popular, when the moisture content of all wooden surfaces was at 37%. While the grinding method did result in an increased recovery of pathogens, there is no scientific evidence showing that entrapped pathogenic bacteria within the porous wooden material can resurface. Another study further investigated the effects of moisture and wooden (maple) and plastic surfaces on *E. coli* growth and persistence and found that the recovery of *E. coli* recovery was greater from surfaces were wet (Aviat et al., 2016). Studies indicate that moisture on the wood surface can assist with bacterial penetration into the porous material. Comparatively, *E. coli* could be recovered from dry plastic surfaces up to 24 hours after initial inoculation.

Additionally, wood may have acquired defense strategies that originated from the intact tree to minimize bacterial invasion and infection. These mechanisms include protective periderm and rhytidome (outer bark) obstructions that prevent the entry of

microorganisms (Aviat et al., 2016; Pearce, 1996). Other defenses against microbial attack include limited availability of oxygen within the wood, limit access to nutrients, and antimicrobial enzymes and compounds. These natural plant defense strategies can be useful when wood is converted to a food contact surface (Canillac and Mourey, 2001). Several studies have been conducted to better understand the antimicrobial properties found in wood, however it is unclear whether these compounds have bacteriocidal or -static properties (Mourey and Canillac, 2002). For example, no inhibitory effect was observed on *Aspergillus niger* with flavonoids and phenolic substances, while others have found a stilbenes, Pinosylvin, and flavonoids inhibit *Aspergillus fumigatus* and *Penicillium brevicompactum* (Välimaa et al., 2007). These results were later corroborated by Plumed-Ferrer et al., (2013) when pinosylvin showed inhibitory effects on *Saccharomyces cerevisiae*. Another stilbene called resveratrol had substantial inhibitory effects on molds and *S. aureus* bacteria found on human skin (Chan, 2002). This applies to artisanal cheesemaking as traditional practices can and often do include direct hand contact with the product during cheesemaking (Uhlich et al., 2006). Yeasts, such as *Candida albicans* and *Saccharomyces cereviseiae*, are inhibited by wood extract and antimicrobial compounds found in wood (Lee et al., 2005). Overall, it seems that antimicrobial compounds may inhibit Gram positive or Gram negative bacteria. Antimicrobial compounds from wood inhibit *L. monocytogenes* and *E. coli* but not coliforms (Canillac and Mourey, (2001). *E. coli*, *Bacillus subtilus*, and *S. aureus* are inhibited by flavones and isoflavones (Chacha et al., 2005). Furthermore, pinosylvin inhibits Gram-positive, including *L. monocytogenes*, *S. aureus*, and *B. cereus*, but excluding lactobacilli, such as *Lactobacillus plantarum* (Plumed-Ferrer et al., 2013). This may be due to the membrane of the bacteria and

lactobacilli may be able to de-polarize the antimicrobial compounds and extracts. The longer those pathogenic bacteria are within the wooden surface, the greater the antimicrobial effect, however this observation only occurred for pine (Schönwälder, et al., 2002).

Microbial mortality plays a major role in recovery rates. Supporting evidence is that recovery rates of *L. monocytogenes, E coli,* and *Penicillium expansum* on surfaces of pine, poplar, and spruce wood decline after 24 hours following grinding, planning, and brushing methods (Ismail et al., 2015). Milling and colleagues (2005) demonstrated that the recovery rate of *E. coli* and *Enterococcus faecium* declined significantly on pine chips when using swabbing methods for detection. After 24 hours, *E. coli* levels fell below detectable levels on pine and spruce chips using standard culturing methods and non-culturing methods. *Enterococcus faecium* was detected on spruce chips but at significantly lower levels than the initial inoculum load. Both, spruce and pine chips, maintained a moisture content of 37% during the first 24 hours after inoculation, providing sufficient evidence that the microbial mortality was not due to declining moisture or wood decay.

In conclusion, *L. monocytogenes* cannot be completely eradicated from processing plants because of it is ubiquitous in nature and there are many entry points that can allow the organism into a facility (Buchanan et al., 2017). This requires food processors to manage trafficking and entry points and, thus, substantiates the need for preventive controls such as environmental sampling plans, good manufacturing practices, sanitation procedures that include breakdown of equipment for cleaning, and other processing measures used to eliminate RTE foods from being implicated in foodborne outbreaks as a result of post-processing contamination (USFDA, 2013).

Potential Transfer of Microorganisms from Cheese Surfaces to Wooden Boards

While the composition of cheese itself such as moisture content, pH, salt content, and affinage conditions minimize the potential for pathogens such as *L. monocytogenes* to survive and persist (D'Amico and Donnelly, 2008), the macro-nutrients (fats, proteins, and carbohydrates) found in cheeses may play a role in microbial survival of bacteria that are transferred to environmental surfaces. While there are no studies that specifically demonstrate transfer of microorganisms from cheese surfaces to food contact surfaces, studies of transfer from foodstuff and other food products onto wooden surfaces has been conducted as a way to evaluate the efficacy of cleaning methods. Residual foods can cause persisting bacteria on food contact surfaces when they permeate damaged or rough surfaces that hold in moisture and create a protective barrier for microbes, such as biofilms (Aviat and others (2016). The efficacy of cleaning and sanitizing plastic and wooden surfaces was tested by enumerating microbes from raw ground beef (Miller et al., 1996, Aviat et al., 2016). The ground beef was in contact with plastic and wooden surfaces for 0, 30, 60, and 90 minutes. After each contact point, these surfaces were cleaned with water or with chemical cleaners, demonstrating no statistically significant differences between surfaces cleaned with water or with chemical cleaner at any time point. The survival of *S. enterica* ser. Typhimurium on plastic and wooden surfaces before and after the application of a sanitizing step in the absence and presence of food residues (Aviat et al., 2016; Gough and Dodd, 1998). Food contact to the wooden surface was applied for 10 minutes and then *S. enterica* ser. Typhimurium was enumerated from rinsing solution used on both surfaces at 30, 60, 90, and 120-minute intervals. Results showed that more bacteria were enumerated

from the rinsing solution used on plastic surfaces than wooden surfaces. However, results also showed that bacterial counts increased on wooden surfaces after 2 hours of contact with microbes. It was concluded that wooden surfaces entrapped more bacteria than plastic surfaces due to stronger attachment. Meanwhile, food residues did not contribute significant differences of *S. enterica* ser. Typhimurium recovery between plastic and wooden cutting boards. Both dishwasher and hand-washing with dish detergent were equally effective at eliminating microbiological risk to remove food contact from a plastic surface or two types of wooden cutting boards (maple and beechwood) (Lücke and Skowyrska 2015 ; Aviat et al., 2016). While the transfer rate after application of foods to wooden food contact surfaces is hard to determine because of the cleaning and sanitizing steps that commenced, there is still the possibility that pathogens from foods can still be detected after cleaning steps (Gough and Dodd,1998).

Potential for Transfer of Microorganisms Embedded in Surfaces to Cheeses

A bacterial population from a given type food product can contaminate another batch of a different food product, by leaving residual bacterial populations on the food contact surface that was used by both food products (Brown et al., 1988, Aviat et al. 2016).

There is demonstrated need to thoroughly clean and sanitize because even short contact times can transfer bacteria from already contaminated surfaces (Dawson et al., 2007; Ismail et al. 2017). Futhermore, food contact surfaces often transmit bacteria to RTE food product during manufacture and transport (Pérez-Rodríguez et al. 2008). The variation of bacterial load that is transferred from the food-processing surface is dependent upon moisture level, contact time, and the amount of pressure applied between food

product and surface. As previously mentioned, the transfer from cheese to surface back to food product may be dependent on the surface type, which include porosity and roughness. In counterpoint, there was no difference in cross-contamination of *Camplobacter jejuni* from wood or polyethylene plastic cutting boards onto cooked chicken (Tan et al., 2011).

Microbial populations found on and within wood surfaces matrices are beneficial to the microbial communities that form on cheeses, such as cooked Ragusano cheese (Di Grigoli et al., 2015) and non-cooked cheeses (Mariani et al., 2011). Transfer from wood to food product has not been thoroughly investigated as of yet (Ismail et al. 2017). Other than cutting board studies, up until Ismail and colleagues (2017) conducted their study, the only other known study that included cross-contamination from surface to food was completed by Montibus and colleagues (2016) who observed the transfer of *E. coli* and *Penicillium expansum* from poplar packaging crates to apples. Nonetheless, transfer rates were low, no more than 0.25%, establishing the integrity of wooden surface is sufficient for a food contact surface.

There was no difference in transfer rates of *L. monocytogenes* from wooden surfaces to young and older cheeses, despite older cheeses having decreased moisture content (Ismail et al., 2017). However, transfer rates from surfaces to young cheeses declined after 15 minutes, and then were stationary up to 24 hours of surface-food contact, which was comparable to other study findings that tested transfer rates of *E. coli* and *Penicilium expansum* (Montibus et al., 2016). This may be due to *L. monocytogenes* entrapment and attachment. Conversely, longer surface-food contact leads to higher transfer rates (Dawson et al., 2007). Discrepancy is likely due to the shorter surface-food contact time intervals used (Ismail et al., 2017). Overall, for both inoculum concentrations

(10^5 and 10^3 CFU/cm^2), *L. monocytogenes* transfer from wooden surfaces to young cheese did not exceed 55%. It was concluded that biofilms may promote the transfer of bacteria to food surfaces, however surface type and porosity may impact the transfer rate from donor surfaces to cheeses.

A U.S. based multi-state listeriosis outbreak occurred in 2012 that resulted 22 cases of foodborne illness, where imported cheeses had been identified as the vector for cross-contamination of *L. monocytogenes* onto other cheeses. This particular outbreak may provide insight into the potential transfer capabilities from a food product to a food contact surface and subsequently to another food product (Heiman et al., 2015). Although the transfer of bacteria resulting in contamination is occurring between two food products, a distributor notified the FDA on August 10, 2012 that *Listeria* spp. was found in the facility after routine environmental testing was completed. In this scenario it could be speculated that transfer from cheese product to food contact surface, and subsequent transfer from surface to cheese product, although the surface type was not specified.

Once again, it is important to take the competitive natural flora of wood into consideration. These microbes include *Lactobacillus bulgaricus, Lactobacillus plantarum* and to a lesser extent by *Lactococcus lactis, Lactococcus cremoris and Streptococcus thermophilus*, which outcompete *L. monocytogenes* for nutrients (Pitt et al. 2000). Many of these LAB are found on the surfaces of wooden vats (Gaglio et al., 2016). Researchers were able to identify LAB such as *Lactobacillus casei, Enterococcus faecium, Lactobacillus rhamnosus, Streptococcus thermophiles*, and *Pediococcus acidilactici*. Biofilms that formed on wooden vats were effectively sanitized as the surfaces tested negative for indicator organisms and pathogens. In agreement, *Salmonella* spp., *L.*

monocytogenes, Escherichia coli, and *Staphyloccocus aureus* (coagulase positive) are unable to adhere to the surfaces of wooden vats because they are outcompeted by starter and non-starter LAB that are essential for cheese manufacture and ripening (Cruciata et al., 2018). These observations occurred on chestnut, cedar, cherry, ash, walnut, black pine, and poplar wood varieties.

Wooden Board Cleaning Practices

Swiss Approach

According to the Swiss Confederation, the use of wooden shelves during cheese ripening has been a traditional practice for decades throughout Europe (Swiss Confederation, 2014). The benefits of using wooden shelving are its ability to retain moisture, resulting in a controlled ripening process. However, due to the porosity of wood, it may pose a food safety risk, as Switzerland had experienced a listeriosis outbreak between 1983 and 1987 that implicated a soft cheese made from pasteurized milk and caused 122 cases, resulting in 31 deaths. Therefore, research was conducted by the Agroscope's Institute for Food Sciences (IFS) to establish a cleaning regimen for cheesemakers who use wooden boards during affinage.

The IFS promulgated standard sanitation operating procedures for cleaning and sanitizing of wooden boards that includes a heating step equivalent to pasteurization. This includes a cleaning step using alkaline detergents at 60°C for a mechanical application, and a subsequent water rinse. Then these shelves are exposed to heat treatment with boiling water or steam using normal pressure in an enclosed area. This is to achieve a temperature above 70°C for 30 minutes, followed by cooling and drying

steps in a contained area. This treatment allows producers to achieve hygienic standards that are required by 21CFR 110.40a as current good manufacturing practices (cGMPs). These heating steps were implemented as a way to eliminate *L. monocytogenes* in the 2mm layer of wood (Zangerl et al., 2010).

French Approach

French standards state that cleaning wooden boards intended for aging cheese with a cold-water soak, followed by mechanically brushing will preserve the natural microflora (Dairy Pipeline, 2002). According to (Mariani et al., 2011), the biofilm ecology of wooden shelves consists of micrococci-corynebacteria (7.2 to 7.3 log10 CFU/cm2) and yeasts/molds (6.0 to 6.1 log10 CFU/cm2). These beneficial bacteria minimize the risk of pathogenic bacteria, such as *L. monocytogenes,* attaching and surviving within the porous surface. Studies have found no pathogens on the surface or within wood, however the French suggest that if wooden boards become heavily contaminated, then they should be discarded (Dairy Pipeline, 2002).

United States Regulatory Policy Regarding Biological Soil Amendments of Animal-Origin (BSAAO)

The FDA Supplemental Proposed Rule for "Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption" has recognized that while biological soil amendments of animal origin (BSAAO) play an important role in providing nutrients to improve soil and produce quality, BSAAO are also a vector for pathogens (Sharma and Reynnells, 2016; FDA HHS 2013). Fruit and vegetables can become contaminated through various conditions and routes prior to harvesting such as

improperly composted BSAAO's, contaminated irrigation water, animals, soil, and dust from concentrated livestock or other animal operations (Beuchat et al., 2001; Cooley et al., 2007; Markland et al., 2013). For example, fruit flies are vectors of *E. coli* O157:H7 to damaged apples under laboratory conditions (Harris et al., 2003). This can be problematic when produce is handled and stored in conditions where flies are difficult to control and damaged produce product is inevitable.

Since the early 1970's, a considerable increase in the consumption of fresh produce has been observed in the U.S (Harris et al., 2003). It is presumed that this is due to the promotion of consuming fruits and vegetables as part of a healthy diet. During the time period between 1982 and 1997, consumption of fresh produce increased from 91.6 kg to 121.1 kg, an increase of 32% per capita. Consequently, per capita fruit and vegetable consumption increased again from 19% to 57% between 1976 and 1997 respectively (USDA, 2008; USDA, 2011; Park et al, 2012).

According to the U.S. Centers for Disease Control and Prevention (CDC), almost half (46%) of all food borne illnesses that have led to hospitalization or death between 1998-2008 were attributable to fresh produce (Painter et al., 2013; Reynnells et al., 2014). Data showed that more illnesses were attributed to leafy vegetables (22%) than to any other food commodity. Illnesses associated with leafy vegetables were the second most frequent cause of hospitalizations (14%) and the fifth most frequent cause of death (6%) (Painter et al. 2013).

Another analysis of outbreaks from 2001 to 2010 completed by the Center for Science in the Public Interest, demonstrated that produce was also the leading single ingredient food commodity to cause the most outbreaks and the most illnesses (CSPI, 2013). Additional data that was reported by the FDA between 1996 and 2010, demonstrated that domestic and imported produce accounted for 23.3% and 42.3% of outbreaks, respectively. During this time frame, approximately 131 produce-related reported outbreaks occurred, resulting in 14,132 outbreak-related illnesses, 1,360 hospitalizations and 27 deaths (FDA HHS, 2013). The majority of fresh produce-related outbreaks and illnesses in the FDA database were associated with bacterial agents (86.5%), followed by parasites (11.6%) and viruses (1.9%). Pathogens of concern include enterohemorrhagic *E. coli* (STEC), *Salmonella*, *Campylobacter jejuni*, and *Cryptosporidium parvum* and *Listeria monocytogenes,* and Hepatitis A. (Sharma and Reynnells, 2016; FDA HHS, 2013). However, *E. coli* O157:H7, *Listeria monocytogenes*, and *Salmonella* are the most important pathogens of concern that attribute to food safety of produce (Park et al., 1972). Increased consumption of raw or minimally cooked fruits and vegetables that are grown and harvested domestically, along with imported produce from other countries that follow lower hygienic standards has caused concern for food safety and public health (Islam et al., 2005).

The economic burden in the United States alone due to foodborne illnesses attributed to *L. monocytogenes*, non-typhoidal *Salmonella*, and *E. coli* O157:H7 is approximately $2.0 billion, $4.4 billion, and $607 million, respectively (Park et al., 2012; Scharff, 2012). The industry costs are derived from expenses on recalled product,

sampling and testing, and preventative action to minimize contamination (Park et al., 2012). The meat processing industry alone expends between $10 million and $24 billion towards *L. monocytogenes* food safety, annually. This applied to the cantaloupe industry when 4,800 cantaloupes were recalled due to the listeriosis outbreak in 2011. This recall also led to a large decline in revenue after consumer demand depreciated. Similarly, the demand for bagged spinach decreased by 43% over the year after the spinach-associated *E. coli* O157:H7 outbreak occurred in 2007. The financial and public health implications provide merit for preventive action to reduce contamination of produce.

According to the FDA, data collected between 2006 and 2010 showed that the following commodities accounted for 88.5% of the total produce-associated outbreaks: (i) 34 outbreaks associated with sprouts; (ii) 30 outbreaks associated with leafy greens such as lettuce and spinach; (iii) 17 outbreaks associated with tomatoes; (iv) 14 outbreaks associated with melons such as cantaloupe and honeydew; (v)10 outbreaks associated with berries, such as raspberries, blueberries, blackberries and strawberries; (vi) 6 outbreaks associated with fresh herbs such as basil and parsley; and (vii) 3 outbreaks associated with green onions (FDA HHS, 2013). Previous studies have shown that produce and produce-containing foods were the second most frequent food source of outbreaks attributed to EHEC during 1982–2002 (Rangel et al., 2005).

Between 2006-2008, surveillance data of foodborne outbreaks within the United States also determined that leafy greens were the leading vehicle of EHEC contamination (Erickson and Doyle, 2007). *Salmonella* spp. was the second pathogen of concern, highly

associated with produce outbreaks of implicated tomatoes and leafy greens (Anderson et al., 2011).

Since 1995, there have been 22 outbreaks that have been associated with raw spinach and lettuce, of which 9 of them were traced to, or near to, the Salinas Valley Region of California (Cooley et al., 2007). The Salinas Valley region is the largest leafy vegetable producer in the U.S, harvesting over 70% of the annual leafy green vegetables crops (Cooley et al., 2007; Moyne et al., 2011; FDA, 2009). Outbreaks include investigations of lettuce and spinach commodities in Washington State during 2002, and two outbreaks in California during 2003, where one STEC strain associated with all three outbreaks was linked to a farm in the Salinas Valley area. The Salinas and San Juan (San Benito County) valleys were also associated with an outbreak of bagged mixed produce, including lettuce, in Minnesota in September 2005 and a multi-state outbreak involving bagged spinach during August/September 2006, respectively.

According to the FDA, the most likely point of contamination for fresh-cut produce is during growing, harvest, packing and holding as a raw agricultural commodity (RAC). When produce is washed or mixed into larger batches, the risk of pathogen contamination becomes greater (U.S. FDA, 2013).

Under FSMA, agricultural water sources are applied to produce during harvest or after harvest directly and indirectly (U.S. FDA, 2011). Indirect scenarios include making agricultural teas, use on food contact surfaces (including ice), and handwashing before and after harvest. Analytical microbiological standards have been promulgated to ensure that water source is not a source of contamination. If any generic *E. coli* is detected in

100 ml of water, corrective actions must be taken to ensure that the water is treated and re-tested before re-introducing it into the harvesting and packaging process of raw produce.

Agricultural water that is applied directly onto produce (except sprouts) must also be tested. Microbiological standards state that 235 CFU (or MPN when appropriate) of generic *E. coli* per 100 ml sample of irrigation water or 126 CFU or MPN) per mean of 5 samples of 100 ml of water is permissible (U.S. FDA, 2018). If standards are exceeded steps will have to be taken to treat and re-test the water source prior to re-application.

The FDA indicated that growers have the option to take an alternative approach for some prescriptive provisions (e.g., standards associated with soil amendments and agricultural water), that are similar to past regulations such as a hazard analysis and critical control points (HACCP) plan for juices as stated under 21 CFR 120.24 (Harris et al., 2012; U.S FDA, 2009). Any alternative approach must be supported by an assessment demonstrating the efficacy of reducing microbial hazards for the described scenario. This would be beneficial to growers to determine when alternatives are most appropriate based on food commodities, conditions, and practices, and can further identify what supporting data is needed.

Pathogen Survival in Soils and Contamination of Produce

Quantitative risk assessments can use die off rates to better identify intervention and preventive strategies to control food safety risks associated with raw produce (D. L. Weller et al., 2017). Survival rates are ideal risk models to estimate potential

contamination levels of produce at time of harvest.

In challenge studies on produce, die off rates of *E. coli* range from 0.4 to 1.64 log CFU/day. One study observed a 0.54 to 1.64 log CFU/day decline on spinach greens in Nova Scotia, Canada (Wood et al., 2010). Die off rates of *Salmonella* in studies conducted in the United Kingdom ranged from 0.43 to 0.76 log CFU/ day on spinach and lettuce. With the more sensitive MPN method, die off rates for *E. coli* are estimated as 0.52 log MPN/day (Weller et al. 2015). Over the 240 hour period between time of inoculation and harvest of lettuce, each head of lettuce sampled had a decline of *E. coli* levels from 8.86 to 3.64 log MPN./day. These results corroborate other reported die off rates of *E. coli* on produce mentioned previously. *E. coli* was still detected 10 days after the lettuce heads were inoculated at 3.64 ± 0.75 MPN. The first 100 hours after inoculation with *E. coli* concentrations between 10^5 to 10^9 CFU/ml is where a significant *E. coli* die off rate occurs (Weller et al., 2017), which is consistent with other studies (Lekkas et al., 2016). Modeling of *E. coli* die off rates on field grown greens, such as lettuce, have been evolving and such models have shown that *E. coli* survival rates follow a rapid decline with a biphasic pattern (McKellar, Lu, and Delaquis, 2002). A linear regression analysis found that the die off rates for lettuce heads were significantly less when harvested after a rain event that occurred 64 to 69 hours after the lettuce heads were inoculated than lettuce harvested prior to the rain event (McKellar et al?).

The possibility of pathogen contamination of produce is possible because *E. coli* O157:H7 persists in soils. *E. coli* O157:H7 can persist in manure amended soils from 154-217 days when inoculated with large concentrations (10^7 CFU/g) (Patel et al., 2010).

The same trial method found that translocation of STEC onto lettuce and carrots continued for 77 days and 168 days respectively after seedlings were planted (Patel et al, 2009; Cooley, 2007). Applied attenuated *E. coli* O157:H7 could be detected on lettuce plants grown in inoculated soil amended with manure composts or by spraying inoculate water once directly onto seedlings, in all treatments at 77 days and in soils through 126 days (Islam et al., 2004). Another study detected *E. coli* O157:H7 7 days after inoculation when the inoculum was applied higher than 6 log CFU/ml (Erickson et al., 2010). *E. coli* O157:H7 persisted on the abaxial surface (underside) of the leaves for longer than the adaxial surface. This suggests dispersal by splashing from soil to leaf surfaces.

The physical environment of produce surfaces can be considered inhospitable for the growth and survival of bacteria, depending upon the lack of nutrients, availability of free moisture, temperature and humidity fluctuations, and ultraviolet light (Harris et al., 2003). Certain conditions, such as sunlight for example, can damage and lyse the bacterial cells due to the short ultraviolet waves. However, free moisture on leaves from various precipitations, such as rainfall, dew, or irrigation, may promote persistence and growth of microbial populations (Park et al., 2012). Many studies confirm that warm temperature, high humidity, and pathogen concentration can affect produce contamination (Dreux et al., 2007; Dreux et al., 2007; Natvig et al., 2002). In comparison to BSAAO, such as manure-amended soils, where pathogen survival declines when temperatures increased between 7°C to 33°C (Park et al. 2012; Semenov et al. 2007; Lekkas et al. 2016), warm temperatures caused growth or maintained pathogens on produce (Park et al., 2012). Demonstrated in vitro, warm growth temperatures (20-30°C)

increased the attachment of *L. monocytogenes* which may also apply to other pathogens. *L. monocytogenes* has the capabilities to carry a gene (*lcp*) that encodes for a *Listeria* cellulose binding protein that may play a large role in attachment onto edible produce (Bae et al., 2013).

High humidity also stimulates growth of *S. enterica* (Dreux et al., 2007; Dreux et al., 2007), and along with high temperatures, increased the attachment of *Salmonella* spp. onto tomatoes (Iturriaga et al., 2003). When higher concentrations of pathogens were applied to soil or water, produce contamination was more regularly identified. However, Park et al. (2012) suggests that there may be a threshold for pathogen concentration required for effective produce contamination.

Consequently, a large concern that exposure to such stressors could result in natural mutation and favor bacteria that can adapt to harsh conditions. Nonetheless, it is unlikely that human pathogens of concern will develop resistance. This is largely due to human pathogens originating from enteric sources so they cannot successfully thrive as plant colonists in comparison to the other hardy plant microbial populations. *E. coli* strains attributed to outbreaks on produce, such as leafy greens, are given the opportunity to adapt to such stressors in the environment pre-harvest, leading to persistence (Markland et al., 2013). Therefore, once in food or food ingredients, *E. coli* O157:H7 has the ability to survive when stored under refrigeration temperatures and has a high acid tolerance (Islam et al., 2004; NACMCF, 1999).

Produce conditions including plant age, leaf age, physical damage and epiphytic bacteria are also correlated with produce contamination (Park et al., 2012). Mature produce intended for harvest is more susceptible to contamination by *Salmonella* and *E.*

coli O157:H7 due to longer bouts of exposure time. However, higher pathogen populations are observed on younger Romaine leaves, suggesting that greater probability of infection and colonization (Brandl and Amundson, 2008; Moyne et al., 2011). Young leaves tend to have high nitrogen content, attractive to bacterial pathogens like *E. coli* O157:H7 (Park et al., 2012). Extensive root systems of mature plants also increase the probability of interaction with pathogens in soil (Mootian, Wu, and Matthews, 2009; Park et al., 2012).

After harvesting, fruits and vegetables are prone to physical damage (i.e., punctures, bruising, or degradation) when handled, possibly stripping the otherwise protective epidermal barrier. This damage on handled or fresh cut produce creates entry points for pathogen infection, especially at non-refrigerated temperatures (Harris et al., 2003; Park et al, 2012). Conversely, pathogens such as nonproeolytic *C. botulinum, L. monocytogenes*, and *Y. enterocolitica* are psychrotrophs that thrive in refrigerated temperatures. *L. monocytogenes* can grow on vegetables under both refrigerated and ambient temperatures and on non-acidic fruits (Harris et al., 2003, Flessa et al., 2005; Ukuku and Fett, 2002). *L. monocytogenes* has also been detected on the surface of tomatoes (Beuchat and Brackett, 1991) and other acidic fruits such as Hamlin oranges (Pao and Brown, 1998) when stored at 20°C. *L. monocytogenes* ability to grow and persist is determined by surface structures and availability of nutrients on fruits and vegetables (Flessa et al., 2005). This was demonstrated when *E. coli* O157:H7 levels declined more rapidly on onions than on carrots, suggesting that antimicrobial phenolic compounds on onions were of higher concentrations than found in carrots (Islam et al., 2004). Freeze-thaw cycles were also reduced pathogen levels on produce (Park et al,

2012). This demonstrates the need for post-harvest processing standards to be based on empirical challenge studies that have assessed risks and best manufacturing practices and preventive controls. For example, one study established that fruits, such as strawberries, are a low risk food for listeriosis because their low pH are not able to support the growth of *L. monocytogenes* (Flessa et al., 2004).

These studies should also reflect natural contamination loads. Most laboratory studies use unrealistically large concentrations of inoculum when research show that pathogens such as *L. monocytogenes* and Shigella producing *E. coli* decline more rapidly at lower inoculation levels (5 log CFU/berry) (Flessa et al. 2004) when compared to larger levels (7 log CFU/berry) (Knudsen et al., 2001; Yu et al., 2001).

Stipulations that require very low microbial counts may be compromising produce safety because the population of non-pathogenic bacteria may be causing a barrier that minimizes pathogen growth and the risk of illness associated fresh cut fruits and vegetables (Harris et al., 2003). Studies have also focused on the impact of epiphytic bacteria on pathogen translocation and survival onto plant tissue (Park et al, 2012). Plant material has approximately 10^5 to 10^7 cells/g of bacteria, being the most abundant microorganism to colonize the phyllosphere, comprising of 10^{26} cells per plant (Morris and Monier, 2003; Williams et al., 2013). Epiphytes on the phyllosphere include *Erwinia* and *Pseudomonas*, however more microbial diversity exists (Delmotte et al., 2009; Kadivar and Stapleton, 2003; Mark Ibekwe et al., 2009; Morris and Monier, 2003; Williams et al., 2013; Yang, et al.,2001). Necrotic lesions formed by epiphytic bacteria provide infection sites and enhance the survival of *E. coli* O157:H7 on plant tissue (Aruscavage et al., 2010). Mechanisms of the epiphytic bacteria may surmount

the plant immune system, allowing these bacterial pathogens to enter through leaf stomata and colonize internal leaf mesophyll. improve internalization of epiphytic bacteria and pathogens such as *S. enterica* into the stomata and leaf tissue. In contrast, epiphytic bacteria within the phyllosphere can also be competitors against pathogens, such as *E. coli* O157:H7 (Park et al., 2012). The outcome of phyllosphere dynamics may be bacteria and plant host species specific. For example, within the lettuce phyllosphere, epiphytic bacteria *Wausteria paucula* promoted the survival of *E. coli* O157:H7 yet *Enterobacter asburiae* had the opposite effect (Cooley et al., 2006). Pathogen establishment within the vascular system for systemic spread is more successful in seedlings than when plants are mature (Jablasone, Warriner, and Griffiths, 2005; Moyne et al., 2011). Leafy green lettuces that were inoculated with *E. coli* O157:H7 at 8 log CFU/ml on the abaxial side of the leaves, had positive detection of the pathogen up to 14 dpi. In comparison, *E. coli* O157:H7 was not detected within the internal portions of lettuce greens when the inoculum was applied to the soil through compost (Erickson et al., 2010).

E. coli O157:H7 levels have been found to declined in the phyllosphere of lettuce. Williams et al., (2013). After 7 dpi, viable cells of *E. coli* O157:H7 were below detection limits for enumeration and only few samples were found positive via enrichment detection methods. Further 454-pyrosequencing was used to identify the bacteria found in lettuce samples harvested at 7, 14, and 21 dpi from four field trials. These samples included treatment groups of lettuce plants under sprinkler or drip irrigation that were inoculated with *E. coli* O157:H7 or left as a control. In total, 652 OTUs were represented through sequencing with a mean of 36 ± 19 OTUs per plant sample, which were found to

be statistically significant through rarefaction analysis. The two dominant bacterial phyla found in the lettuce phyllosphere were *Proteobacteria* and *Firmicutes* and statistical analyses showed that they were negatively correlated. Other phyla that were present but only comprised of less than 1% of the sequences inspected included *Bacteroidetes, Deinococcus-Thermus, Acidobacteria, Gemmatimonadete*, TM7, and *Nitrospira.* The majority of sequences fell under the *Enterobacteriaceae* family, where *Pantoea, Leuconostoc, Pseudomonas*, and *Erwinia* were abundant genera found. Interestingly, *E. coli* was infrequently found on plants and only amounted to under 0.0001% of total sequences completed. Of lettuce samples tested, the majority of sequenced DNA was traced to members of the *Enterobacteriaceae* family, comprising a total of 86 OTU's. Classification of sequences identified that six OTU's were to *Enterobacter* sp., three to *Erwinia* sp. and one OTU each was associated with *Tatumella, Citrobacter, Raoutella, Brenneria*, and *Panoea* sp. This study also found that bacterial diversity on lettuce was similar to what was found in field trials and was varied based on season of planting (early versus late). *Panteo, Pseudomonas, Erwinia*, and *Enterobactericeae* Clade 1 were amount the most abundant microbial genera in the phyllosphere during the late season lettuce plants. Early season plant phyllospheres were colonized by abundance of *Leuconostoc, Lactococcus, Bacillus,* and *Exiguobacterum* within the *Leuconostocaceae, Bacillaceae*, and *Streptococcaceae* families.

The effects of irrigation on the plants revealed that the microbiota differed between plants that were drip versus sprinkler irrigated for eight out of the twelve sampling points (Williams et al., 2013). *Erwinia* and *Xanthomonas* species were detected for drip and sprinkler irrigation, respectively. Overall, the microbial diversity of the plant

phyllosphere is limited in comparison to other soil and marine environments (Delmotte et al., 2009; Telias et al., 2011; Williams et al., 2013). This study also found that efficacy of standard laboratory culturing methods was limited and could only grow 1-10% of the total cells on the lettuce samples. Several studies have shown that *Proteobacteria*, *Firmicutes*, and *Actinobacteria*, were the most prominent bacterial phyla found on Romaine lettuce (Knief et al., 2011; Rastogi et al., 2012; Williams et al., 2013), although *Firmicutes*, including lactic acid bacteria (LAB), was newly found as another abundant portion of the microbiota. Plants where low levels of viable *E. coli* O157:H7 cells survived and persisted after inoculation created a very distinct microbiota on lettuce plants and contributed to a lower total cell count. Williams et al. (2013) also found that the presence of *Erwinia* is likely to be associated with a decrease in the presence of *E. coli* O157:H7 on plants due to competition for nutritional resources.

Previous literature reports have shown that *E. coli* O157:H7 populations decreased over 15 days at 4°C on spinach washed with tap water. Aerobic microbial counts increased over time in a packaging environment that consisted of gas-permeable films with oxygen transmission rates (OTR) of 110 cc O_2/100 in^2/24 h and with 40 microperforations, while inoculated *E. coli* O157:H7 counts decreased on shredded lettuce (Sharma et al., 2011). Packaging was also flushed with N_2 to achieve an O_2 level of 2% once vacuum-sealed. The growth of aerobic bacteria under those treatment conditions indicate that the increase of these psychrotropic consortia influenced the reduction of *E. coli* O157:H7 on shredded lettuce (Sharma et al., 2011). Similarly, *E. coli* O157:H7 counts marginally declined on Romaine lettuce after being stored for 9 days at 15°C under atmospheric conditions (Sharma et al., 2011; Carey, Kostrzynska, and

Thompson, 2009). Moreover, packaged chopped or shredded lettuce can support the growth of *E. coli* O157:H7 when storage temperatures exceed 8°C (Moyne et al., 2011; Abdul-Raouf, Beuchat, and Ammar, 1993). This is also the case for *L. monocytogenes* that declined on the surface of strawberries after 4 to 7 days when storage temperatures were reduced from 24°C to 4°C (Flessa et al., 2004).

Recent attention has been drawn to the possible internalization of *E coli* O157:H7 in tissues of produce, which would make it difficult to eradicate the pathogen by washing (Sharma et al., 2009). A Green Fluorescent Protein (GFP) labeled STEC O157:H7 was applied to spinach that was grown hydroponically or in pasteurized soil (Sharma et al.,2009). The GFP protein fluoresces under UV light. . There was evidence of internalization into spinach plants grown in hydroponics but not pasteurized soil.

Why internalization could occur with this medium and not soils is unknown. It is speculated that the medium may have allowed motile *E. coli* O157:H7 to traverse through and internalize in the root hairs. This also signifies that the cells were under less physiological stress in the hydroponic medium in comparison to the pasteurized soils. The cause for greater internalization of spinach from hydroponic media may be due to the decreased competition for nutrients in the system, allowing pathogens that have a physiological fitness advantage to colonize and internalize into plant tissues. These results are also comparable to other studies where *S. enterica* ser. Typhimurium (but not *E. coli* O157:H7) internalized into lettuce seedlings grown in hydroponic conditions after 3-6 weeks of growth (Herman et al, 2008). The difference in results may be due to the different formulations of hydroponic media used. Regardless, this study further supports what Sharma et al. (2009) hypothesized and suggests that Hoagland's hydroponic agar

may be a better medium to observe *E. coli* internalization into roots of leafy greens. It also needs to be considered that plants may carry low levels of environmentally stressed *E. coli* O157:H7 and further research is warranted (Moyne et al., 2011).

The expression of virulence factors is differentiated in bovine colonization and human infections (Sharma et al., 2011). On leafy greens, stx_2 and intimin (eae) gene expression was upregulated slightly on Romaine lettuce inoculated with an *E. coli* O157: H7 strain that expressed stx_1 and stx_2 genes, when stored at 4C for 9 days and exposed to atmospheric conditions (Carey et al., 2009). Virulence could be affected and potentially improved when persisting on iceberg lettuce under a variety of packaging conditions (Sharma et al., 2011).

Under packaging O_2 conditions of 2%, inoculated *E. coli* O157:H7 had increased expression of stx_2 on lettuce, which may lead to more Shiga toxin production, and greater risk of foodborne intoxication regardless of host colonization (Sharma et al., 2011). These same conditions also caused intimin *eae* gene expression to increase by two-fold. This suggests that *E. coli* O157:H7 has greater potential for attachment and colonization on the intestinal wall and the formation of effacement lesions in the host. Similarly, virulence factors encoded by *rfbE* were also upregulated under the same conditions. Studies have shown that *E. coli* O157:H7 mutant that lacked the *rfbE* gene did not persist as long as the wild-type, signifying that *rfbE* may also play a role in attachment and colonization onto host intestinal epithelial cells.

More observational studies should be conducted to assess the risk factors that are associated with produce contamination in a more natural environment (Park et al., 2012). Additional experimental challenge studies are needed to provide evidence to support

guidelines for preventive controls on produce contamination. There is a large pool of literature focusing on produce contamination by foodborne pathogens of concern, only a few of them promulgated findings that evidenced causality when determining produce contamination (Park et al., 2012). Based on existing literature, reducing microbial contamination of soil or irrigation water are the best approaches towards minimizing and preventing produce contamination (Park et al., 2012).

Impacts of drip versus overhead sprinkler irrigation were evaluated for their effect on the persistence of attenuated *E. coli* O157:H7 in the lettuce phyllosphere grown in the Salinas Valley region of California (Moyne et al., 2011). The percentage of plants that were *E. coli* O157:H7 positive was always higher during the spring 2008 trial when compared to the spring and fall 2009 trials. There was an inverse relationship between abundance of *E. coli* O157:H7 and indigenous bacteria. lettuce plants that were irrigated by overhead sprinklers had greater populations of indigenous bacteria than plants receiving drip irrigation. *E. coli* O157:H7 could not be retrieved from lettuce plants when the soil was inoculated prior to seedling germination, and at 28 days after 2-week-old lettuce plants were inoculated. *E. coli* O157:H7 was detected at 2 days after 4 week old lettuce plants were inoculated through enrichment for few samples. By day 7 dpi, counts for 82% of lettuce plants were below detectable levels. To further assess data, Moyne et al. (2011) compared their findings to a field study on *E. coli* and *Pseudomonas syringae* (a plant-associated bacterium) had similar growth trends in wet conditions, but in dry conditions *E. coli* significantly declined, while *P. syringae* persisted (O'Brien and Lindow, 1989).

Similar to Moyne et al. (2013), other studies report sprinkler irrigation often led to more bacterial growth on lettuce than drip irrigation that was attributed to greater availability of free water (Williams et al., 2013).

The FDA makes no exceptions from what is required by the USDA National Organic Program (Sharma and Reynnells, 2016) for BSAAO (U.S. FDA,2015). Regulations stem from research suggesting that *E. coli* O157:H7 can survive in harsher environmental conditions than initially determined (Islam et al., 2004). This is supported by investigation of an outbreak of *E. coli* O157:H7 linked to apple cider in eastern Massachusetts (Besser et al., 1993). Manure was used as a fertilizer in an orchard where *E. coli* O157:H7 was detected in apple juice refrigerated for 20 days at a pH below 4.

Initially, in 2011, the Food Safety Modernization Act proposed an interval between BSAAO application and harvesting crops of 9 months (270 days) (Lekkas et al. 2016). This proposed rule was based off of a study conducted by Islam et al (2004), where *E. coli* O157:H7 was able to persist in plant-cultivated soils for up to 7 months in southern fall/winter conditions.

However, the FDA has put the regulations on hold until emperical data are available to better inform their policies. For now, FDA utilizes the USDA National Organic Program standards where manure-amended soils must have a 90/120-day withholding interval before crops can be harvested and compost must meet temperatures of 131°C for at least 3 days, with a 45 day curing interval, before soil application (Islam et al., 2004; Reynnells et al., 2014; Sharma et al., 2016). Research has since been completed to further investigate the survival of *E. coli* in BSAAO amended soils. Rate of manure application was also found to not impact decline rates of *E. coli* O157:H7.

E. coli O157:H7 declined in manure-amended soils stored at 4°C more rapidly than soil stored at ambient temperatures (Jiang et al., 2002 and Mukherjee et al., 2006). Miller and Berry, (2005) revealed that *E. coli* O157:H7 levels were unchanged or increased at all but the lowest moisture level (0.11 g H2O g^{-1}). There is validation that soil moisture and pH do not impact *E. coli* survival (Çekiç et al., 2017). Several studies have shown that *E. coli* death rates are greatly impacted by temperature (Semenov et al., 2007; Sivapalasingam et al., 2004), where some studies have found that increased temperatures or freeze-thaw cycles can cause a decline in microbial population levels (Lekkas et a., 2016; Jamieson et al., 2002; Zaleski et al. 2005). o STEC O26 survived for an extended period of time under very dry conditions (92% dry matter) ; Jiang et al.,(2002).

To better determine the variation of *E. coli* O157:H7 survival, field studies were completed under an USDA grant at the University of Vermont to represent the northeastern region of the U.S. Lekkas et al. (2016) found that manure had no significant effect on the survival Rif^R *E. coli* during the field study, however tillage versus surface application did have an effect on Rif^R *E. coli* populations. Both sites with plots that were surface amended with dairy manure declined below detectable levels after day 14. Tilled plots with no dairy manure amendment in both sites had greater levels of persisting Rif^R *E.coli,* than plots not tilled. Overall, results showed that after 135 and 165 dpi, Rif^R *E. coli* could not be detected via enrichment in both sites.

Challenge studies continue to be essential to determine how the FDA's suggested withholding interval prior to edible crop harvest will be established, due to the potentially negative economic impact on farmers (Çekiç et al., 2017; Sharma et al., 2016).

827 million tons of compostable materials are used annually, primarily for agriculture, sewage, and industry purposes (Barker et al., 1997). Out of these materials, 140 million tons (17%) are collected for the use of composting (Ahmad et al., 2007). Compost is a form of BSAAO that is defined as organic material that has become a nutrient stabilized, humus-like material that reaches thermophilic temperatures (>55°C) to kill any bacterial pathogens (Partanen et al. 2010; Reynnells et al., 2014). This is assuming that adequate C:P:N ratios have been achieved, along with proper aeration and moisture (Reynells et al. 2014; Ahmad et al., 2007). During a typical composting process, bacteria and fungi are expected to be present and are essential for proper composing to be achieved (Partanen et al. 2010). Major bacterial groups in the beginning stages of composting are mesophilic organic acid producing bacteria such as *Lactobacillus* spp. and *Acetobacter* spp. During the thermophilic stages, Gram-positive bacteria such as *Bacillus* spp. and Actinobacteria are most prevalent. During the composting process, bacteria may be active cells, dormant cells, or be present as spores. A diverse microbial population based on the number of Operational Taxonomic units (OTU's) is present during the initial composting stage due to little competition for nutrients. This competition increases as nutrients become utilized throughout the cycles of composting. Particle size of the composting material overtime affects the oxygen movement, therefore impacting the microbial community composition by limiting microbial and enzymatic access to substrates (Ahmad et al., 2007; Zaleski et al., 2005). Too small of a particle size can create an anaerobic environment within the composting material and too large of a particle size may stop the process completely.

Achieving correct composting standards is important to eliminate harmful pathogens (Ahmad et al., 2007; Lemunier et al., 2005; Reynnells et al., 2014). Achieving thermophilic parameters in compost is crucial to avoid exposing foodborne pathogens to becoming acclimatized or "heat shocked" during the mesophilic stage at sublethal temperatures (45-50°C). This may allow pathogens to survive during the thermophilic stage of the composting process (Reynnells et al., 2014; Singh, Jiang, and Luo, 2010). This is a safety concern since pathogen regrowth presents a risk for produce contamination when amended in soils intended for growing and harvesting crops. *Salmonella* spp. and *E. coli* are most susceptible to regrowth in compost when other indigenous microbial communities have already been eliminated from during the composting process (Reynnells et al., 2014; Sidhu and Beri, 1989) . The C:N ratio and moisture content contribute to the regrowth of pathogens in finished compost when thermophilic parameters are not achieved (Reynnells et al. 2014). *S. enterica* ser. Typhimurium and avirulent *E. coli* O157:H7 survived for 7 and 16 days longer during the composting process, respectively (Singh, et al., 2010). The STEC strain (*E. coli* O157:H7 F06m-0923-21) associated with a spinach outbreak survived during a longer period of time at 50°C, 55°C, and 60°C than other STEC strains (Singh et al., 2011). The level of acclamation for *L. m.* during the mesosphilic stage of the composting process was dependent on the bacterial strain, type of heat shock, and type of stressors (Singh et al. 2010).

Soil Amendments and Indigenous Microbial Communities

The produce rule specifies that biological amendments that undergo a physical (thermal), chemical, or combined process must achieve the microbial standards whereby

L. monocytogenes cannot be detected using a method that identifies 1 CFU/5 grams of analytical portion, *Salmonella* spp. cannot be detected above 3/MPN per 4 grams of total solids dry weight, and *E. coli* O157:H7 cannot be detected above 0.3 MPN per one gram analytical portion (U.S. FDA, 2018). Under the Proposed Produce Rule §§ 112.54(b) and 112.55(b) subparts, it is stated that biosolids must contain <1000 most probable number (MPN)/g for BSAAO that are treated (FDA, 2018).

Compost is promoted as a safer and more sustainable approach in comparison to raw manure (U.S. FDA, 2018). The FDA has even considered eliminating the 45 day application interval for composts that are treated properly, with the understanding that compost is a BSAAO that proposes less of public health risk (Lekkas et al. 2016; NSAC, 2014).

The FDA recommends application of FSMA-compliant compost to soils for production of fresh produce instead of manure to reduce risk of pathogen contamination on the harvested produce when other stipulations in the Produce Safety rule are also implemented. However, the source of compost has yet to be distinguished by the FDA.

Compost is intended for use as a biological soil amendment that still provides a nutrient dense biosphere that can drive nutrient cycles and composition essential for crop health (Sinsabaugh and Shah, 2012). Heterotrophic microbes are essential for the biogeochemical cycles that mineralize dead organic matter to the essential elements (Moorhead et al., 2016). The production of organic molecules is an intracellular process where compounds are generated from precursors consuming carbon (C) from the environment. However, once these catabolic enzymes are released into the extracellular environment via lysis or secretion, they activity and turnover of these substrates play key

roles in physicochemical and biochemical interactions. Decomposition is considered a rate-controlling step in the microbial consortia cycle of C and is essential to continue the ratio of C: phosphorus (P): nitrogen (N). Studies have determined that the four extracellular enzymes used to measure C: N: P ratios are β-1, 4-N-acetylglucosaminidase (NAG), β-1,4-glucosidase (BG), leucine amino-peptidases (LAP), and acid/alkaline phosphatases (AP) (Moorhead et al., 2015). Sinsabaugh et al. (2008) reported a junction of In(BG)/In(NAG+LAP) and In(BG)/In(AP) ratios of 1:1:1 for C:N:P enzymatic activities in soils from various ecosystems. The largest source of organic C is from structural polysaccharides that create the cell walls and matrix glycosolates of plants and other microorganisms, along with lignin and other secondary polyphenolic molecules, lipids and storage polysaccharides (Sinsabaugh and Shah 2012). The degradation of polysaccharides is primarily hydrolytic, while phenolic and lipid degradation is primarily oxidative. Organic N is found from polymers of amino acids and aminosaccharides, which have both C and N. P is found in labile nucleic acids, and unruly storage products such as inositol phosphates. With ecoenzymatic activity (EEA), small products are created and can be degraded by microorganisms, such as α- and β-1,4-glucosidase (Sinsabaugh and Shah, 2012). LAP is one of many protease/peptidase enzymes that assist in the cleavage of amino acids from proteins and peptide substrates (Moorhead et al., 2015). These low-molecular mass products are what catalyze the cleaving of cellobiose to glucose (glucans) and leucine and alanine aminopeptidase, which hydrolyze the two most abundant amino acids in polypeptides, β-1, 4-N-acetylglucosaminidase as part of the N terminus. NAG catalyzes the hydrolyzing of oligomers in N-acetyl glucosamine (amino sugar) in chitin and phosphates that can be found in peptidoglycan,

fungal cell walls, and invertebrate exoskeletons of bacteria (Moorhead et al., 2015; Sinsabaugh and Shah, 2012). This further catalyzes the reaction to convert phosphoesters to phosphate. Often times, cell surfaces and periplasmic spaces are where the enzymes that catalyze the terminal reactions in polymer degradation are located. Measuring EEA has been completed by measuring the increase of phosphatase activity in response to N fertilization, demonstrating that microbial communities allocate resources in relation to what nutrients are available in the environment. The understanding of enzyme activity was initially discussed by Overbeck (1991), summarizing the history of aquatic enzyme research with findings from a 1906 paper on proteolytic activity in surface water. According to Burns and Dick (2002), research on aquatic and soil ecology have since converged to facilitate comparisons using concepts such as the existence of biofilms. Furthermore, within the past decade EEA research has evolved to a biomics paradigm, which describes ecological communities as metagenomes and metaproteomes (Sinsabaugh and Shah, 2012). The most studied EEA is the inverse relationship that researchers have observed between phosphatase activity and P available in the environment (Sinsabaugh and Shah, 2012). In soil metabolism, β-glucosidase activity is correlated positively with microbial metabolism and degradation of vegetation (Allison and Vitousek, 1998; Sinsabaugh and Moorhead, 1994; Sinsabaugh and Shah, 2012). However, biotic and abiotic conditions can impact and modify the availability of nutrients and ecoenzymatic secretion (Caldwell, 2005; Cutler et al., 2018; Mark Ibekwe et al., 2009).

Composition of microbial communities in soil is visualized through ordination, a term that was coined by Goodall in 1954 as “an arrangement of units in a uni- or multi-

dimensional order" (Whitmore, 2012). Although this taxonomy theory was applied to upland forest communities in a study completed by Bray and Curtis, (1957), the same model applies to microbial communities. These classifications (i) correlate species with a specific host or the environment, (ii) explore community groupings by establishing groups of species with highest inter-specific correlations; and (iii) to determine the degree of overlap between family, genera, or species as a way to describe variety within a community (Bray and Curtis, 1957).

Availability of nitrogen is highly correlated to *E. coli* survival (Cutler et al., 2018; Franz et al., 2008). In fresh manure, 60-80% of N is typically in an organic form, such as urea and protein (Kelleher et al., 2002), where the 40-90% of this organic N will be converted to ammonia within a year, depending on environmental variables. Another factor that may need to be assessed is the types of soil that may be mixed with the compost, as this has correlated with survival and persistence of pathogens over time. Sandy soils tend to hold less moisture when compared to loamy soils (Fremaux et al., 2008; Fremaux, Prigent-Combaret, and Vernozy-Rozand, 2008; Locatelli et al., 2013; Sharma et al., 2016). Reynnells and colleagues (2014) investigated detection methods for *Salmonella* and *E. coli* O157:H7 and their regrowth potential in composts. They had taken geographical variation into account and completed the same design in several states.

Research has also found that the survival of *E. coli* O157:H7 relied on soil type, where persistence of *E. coli* in sandy soils was due to a larger concentration of organic carbon or biomass carbon while microbial diversity and organic nitrogen has a direct effect on *E. coli* survival in loamy soils. Another study also suggested that soil type affects persistence of *E. coli*, where a field study in Canada compared *E. coli* survival in dairy

manure-amended soils (loamy and sandy). There was no significant difference in the decay rate of *E coli* O157:H7 in both soil types, suggesting that the environmental biotic and abiotic parameters did not support growth (Sharma et al., 2016). However, another study demonstrated that there was no significant difference in decay rates of *E. coli* O157:H7 populations in two different soil types (Sharma et al. 2016). This is possibly due to moisture, stressing the importance of abiotic C:N ratio. Sharma et al. (2016) also discusses the higher nutrient content (N:P) in poultry litter amendments attributing to the extended survival of generic *E. coli* and O157:H7.

L. monocytogenes is a pathogen of concern that has been found in decomposing plant material and manure and is often linked to produce outbreaks (Hutchison et al., 2004; Jiang et al., 2002; Nightingale et al., 2004; Park et al., 2012). *L. monocytogenes.* is recognized as one of the most important food-borne pathogens and can cause listeriosis outbreaks that may attribute up to 30% of mortality rates in immunocompromised populations (Locatelli et al., 2013). L. m can be found in vegetation, water, sediment and soil. *L. monocytogenes* can colonize mammals and is more commonly found in cattle (33%) than sheep (8%) or swine (5.9%). This has led to translocation of *L. monocytogenes* from amended soils onto produce and seeds of crops such as carrots, lettuce, radish, spinach, and tomato.

Long-term *L. monocytogenes* survival also depends heavily on soil texture and clay content, surviving up to 84 days in 71% of tested soils (Locatelli et al., 2013). This has led to translocation of *L. monocytogenes* from amended soils onto produce and seeds of crops such as carrots, lettuce, radish, spinach, and tomato (Locatelli et al., 2013), due to its presence in vegetation, water, sediment and soils. *L. monocytogenes* will persist for longer

durations of time in a fertile soil when compared to a clay soil. However, the pathogen was more abundant in clay soils when compared to sandy soils (Locatelli et al., 2013).

Intrinsic edaphic factors within soils and extrinsic environmental factors influence the survival and persistence of *E. coli*, *Listeria* spp. and *Salmonella* populations (Cutler et al., 2018; Pugliese et al., 2008; Van Elsas et al., 2011). Competition between pathogens and indigenous microbial soil consortia has been observed in several studies (Park et al., 2012).

Previous studies have investigated the survivability of pathogens in manure-amended soils. However, these studies applied large population levels of pathogens or indicator organisms, which are not realistic conditions (Jiang et al., 2002). It is more realistic to test impact of compost and manure amended-soils on *Enterococcus* and *C. perfringens*, which were applied at levels of 1,000 CFU/g and 100 MPN/g respectively (Brochier et al., 2012).

Studies have attempted to establish generic *E. coli* as an indicator for presence of pathogen contamination in soils and water sources intended to come into contact with produce (Cooley et al., 2007; Natvig et al., 2002). Indigenous populations of *E. coli* in soils exceeded those of *S. enterica* serovar Typhimurium, suggesting that generic *E. coli* is a useful indicator organism for evaluating risk of vegetable contamination with BSAAO (Natvig et al., 2002). To determine a correlation between generic *E. coli* and *E. coli* O157:H7, water samples from Salinas Valley were analyzed for coliforms and generic *E. coli* to determine the total maximum daily load (TMDL). Results showed that the Salinas Valley watershed would not meet hygiene standards as demonstrated by >10% of samples exceeding the coliform and *E. coli* standard of 400 MPN/100 mL

(Cooley et al., 2007). After researchers analyzed data from the California Water Quality Control Board, it was established that there was a correlation between the incidence of *E. coli* O157:H7 and generic *E. coli* levels found in watershed samples. However, there was no significant correlation from individual sample sites due to a low incidence of O157:H7. According to the report, *E. coli* O157:H7 was undetected in many samples with high generic *E. coli* levels, suggesting that generic *E. coli* is a poor indicator of *E. coli* O157:H7 presence.

Limitations of 16S rRNA Gene Sequencing of Microbial Communities

While 16S rRNA gene sequencing is a well-known tool to determine microbial communities that are present in various matrices, there are limitations to the method. While, the amplification of 16S rRNA genes is effective at capturing broad shifts of microbial community diversity over time, there are known biases that produce differences in diversity when compared to the metagenomic approach (Poretsky et al., 2014).

The overall aim of this dissertation was to conduct research that would better inform food safety and public policy through the assessment of food safety risk and comparative evaluation of materials available to the food industry.

CHAPTER 2: FDA's Cheese and Cheese Products Compliance Program Guideline Criteria for Non-Toxigenic *Escherichia coli*: A Retrospective Analysis of Impacts on Domestic and Imported Cheeses

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Abstract

The U.S. Food and Drug Administration's (FDA) 2015 Domestic and Imported Cheese and Cheese Products Compliance Program Guidelines (CPG) (U.S. FDA, 2015) consider cheeses to be adulterated if non-toxigenic *Escherichia coli* (*E. coli*) levels of greater than 10 most probable number per gram (MPN/g) and less than 100 MPN/g are found in 3 or more of 5 subsamples. It is unclear if, or how, these standards impact food safety, and the extent to which these standards affect domestic and imported cheese commerce. We conducted a retrospective analysis of microbiological data from FDA's Domestic and Imported Cheese Compliance Program for cheese samples collected between January 1, 2004 and December 31, 2006. Out of 3,007 cheese samples tested by the FDA for non-toxigenic *E. coli,* 76% (2,300) of samples contained *E. coli* levels that exceeded 10/g. Of these samples, 68% (2,047) exceeded 2009 regulatory guidelines of 100/g. In comparison, only 7.7% (232) of tested cheese samples exceeded European Union (EU) standards (<1,000 *E. coli*/g) and 170 (5.7%) of samples exceeded the 1998 CPG criteria (<10,000 *E. coli*/g). Mexican-style soft, semi-soft, and soft ripened cheeses were the cheese types most impacted by application of the 2015 non-toxigenic *E. coli* standards. At *E. coli* levels of 10/g and 100/g, there was no statistically significant association with the presence of *Listeria monocytogenes* or *Salmonella*. However, associations between *S. aureus* levels of 10,000 CFU/g and presence of *Salmonella* and *L. monocytogenes* were statistically significant, indicating that EU regulations targeting *S. aureus* as the pathogen of concern may be more appropriate for cheese safety assessment.

Introduction

U.S. artisan cheeses, along with European Protected Designation of Origin (PDO) and Appellation D'origine Protégée (AOP) cheese varieties imported into the United States are facing unprecedented regulatory challenge. Issues such as the 60 day aging rule (U.S. FDA, 2006); the soft cheese risk assessment (Health Canada and U.S. FDA, 2015); *Listeria* surveillance, wooden shelves for cheese aging (U.S.FDA/CFSAN, 2014); the Food Safety Modernization Act (FSMA); and non-toxigenic *Escherichia coli* (*E. coli)* standards (Correll, 2014; U.S. FDA CFSAN, 2016) are but a few of the regulatory issues confronting cheese makers.

The FDA initiated the Domestic and Imported Cheese and Cheese Products Compliance Program (DICCP) in 1998 (D'Amico and Donnelly, 2011). The purpose of the program was for the FDA to conduct inspection of domestic cheese firms, and to examine samples of imported and domestic cheeses for microbiological contamination, phosphatase and filth. Filth is defined by the Food, Drug and Cosmetic Act as "contaminants such as rat, mouse or other animal hairs and excreta, whole insects, insect parts and excreta, parasitic worms, pollution from the excrement of humans and animals, as well as other extraneous materials which, because of their repulsiveness, would not knowingly be eaten or used" (Olsen et al. 2001). Each cheese sample was analyzed for six attributes, which included: 1. *Listeria monocytogenes*, 2. *Salmonella*, 3. *E. coli* and Enterotoxigenic *E. coli* (ETEC), 4. Enterohemmorrahagic *E. coli* (O157:H7), 5.

Staphylococcus aureus, and 6. Phosphatase. ETEC analysis was required only when *E. coli* was present at ≥10,000 CFU/g (D'Amico and Donnelly, 2011).

The International Commission on Microbiological Specifications for Foods (ICMSF) is recognized as the leading global scientific body for establishment of microbiological criteria in foods. Europe considered ICMSF guidance in establishment of microbiological criteria for cheese in EU Regulation 2073 (2005). In Book 2 (ICMSF, 1986), its risk assessment for cheese, ICMSF writes:

"While the coliform problem in cheese is well known, presence of these organisms in many cheese varieties is extremely difficult to prevent completely. With some varieties, if coliforms are present initially, it is virtually impossible to prevent their growth during manufacture or during the ripening period. In several types of cheese *E. coli* can even be considered characteristic. With the exception of some strains of *E. coli* high populations of coliforms are unlikely to present a health hazard. There is ample evidence that if pathogenic strains of *E. coli* (PEC) are present early in the cheesemaking process their numbers may increase to hazardous levels. However, in view of the scarcity of evidence of recurring outbreaks due to PEC in cheese and the high cost of routine testing, it is doubtful that establishment of end-product criteria for either coliforms or *E. coli* would be justified. Accordingly, no sampling plan is proposed."

EU Microbiological criteria for cheese are risk based and differ depending upon whether cheese has been made from heat treated versus raw milk. In cheese made from heat treated milk, limits have been established for *Staphylococcus aureus* (food safety index), along with targets for *E. coli* (hygienic index) (EU, 2005). The application of *E. coli* limits provides a scientifically meaningful standard in cheese made from heat treated

milk as *E. coli* will not survive heat treatment, thus its presence in cheese made from heat-treated milk indicates post-process recontamination where n=5, c=3, m=10 and $M=10^2$ (Table 1) (ICMSF, 2011). For cheeses made from raw milk, a sampling plan targeting coagulase positive *S. aureus* was established, where n=5, c=2, $m=10^4$ and $M=10^5$ (Table 1). The stage of cheese making where the criterion applies is "at the time during the manufacturing process when the number of staphylococci is expected to be the highest." Action required in the case of unsatisfactory results includes "improvements in production hygiene and selection of raw materials. If values of $>10^5$ CFU/g are detected, the cheese batch has to be tested for staphylococcal enterotoxins." It is notable that no limits were established by the EU for *E. coli* in raw milk cheese. *E. coli* does not offer a meaningful hygienic index in raw products as its presence is expected, consistent with guidance from ICMSF (ICMSF, 2011). In conflict with European Union (EU) and International Commission of Microbiological Specifications of Foods (ICMSF) guidance, the FDA revised the DICCP in its 2009 Compliance Policy Guide (CPG), stating "The presence of *Escherichia coli* in a cheese and cheese product made from raw milk at a level of greater than 100 MPN/g indicates insanitary conditions relating to contact with fecal matter, including poor employee hygiene practices, improperly sanitized utensils and equipment, or contaminated raw materials" (U.S. FDA CFSAN, 2009).

The 2009 CPG document was made available for public comment and FDA received 4 comments, one of which was from the American Dairy Products Institute, who stated "in our view, the permissible level of *Escherichia coli* should be set according to standards of food safety without regard to the treatment of the milk itself. Stated another

way, the guidance should be set at a uniform level to ensure food safety across all covered dairy products" (U.S FDA/HHS, 2014).

In response, FDA issued 2010 CPG Guidance, stating that for non-toxigenic *E. coli*, "Dairy products may be considered adulterated within the meaning of section 402(a)(4) of the Act (21 U.S.C. 342(a)(4), in that they have been prepared, packed or held under insanitary conditions whereby they may have become contaminated with *E. coli* levels greater than 10 MPN per gram in two or more subsamples or greater than 100 MPN per gram in one of more subsamples" (U.S. FDA CFSAN, 2010).

This guidance was subsequently revised, and on July 30, 2015, the FDA reissued the Domestic and Imported Cheese Compliance Program guidelines (U.S. FDA, 2015). In the new guidance, FDA established a 3-class sampling plan for limits on *E. coli* in domestic and imported cheeses (n = 5, c = 3, m = 10 MPN/g, M = 100 MPN/g). If *E. coli* levels exceed 10 MPN/g but are less than 100 MPN/g in three or more subsamples, or greater than 100 MPN/g in one or more subsamples, the cheese is considered adulterated.

Previous work by D'Amico and Donnelly (2011) analyzed FDAs Domestic and Imported Cheese Compliance Program results from January 1, 2004-December 31, 2006 to determine the incidence of bacterial pathogens (*Salmonella*, *E. coli* 0157:H7, *S. aureus* and *L. monocytogenes*) in tested cheese samples. These authors found that out of a total of 3,360 cheese samples analyzed for *E. coli* O157:H7, only 3 (0.08%) cheese samples tested positive. Of the 2,181 samples tested for *L. monocytogenes*, only 52 samples (2.4%) were found positive. *Salmonella* was detected in 45 of 3520 (1.3%) samples. *S. aureus* was present in 135 (6.9%) of 1,600 total cheese samples tested and was the most

commonly detected pathogen. Overall, the low incidence of these pathogens of concern in cheeses questions the need for the revised guidance.

Through correspondence with the American Cheese Society (ACS, 2014; Correll, 2014), the FDA concluded that M at 100 MPN/g is consistently attainable. The objective of this study was to assess the impact of the FDA's 2015 Compliance Program non-toxigenic *E. coli* criteria on domestic and imported cheeses by performing a retrospective analysis of *E. coli* results obtained from FDAs Domestic and Imported Cheese Compliance Program for cheese samples tested between January 1, 2004 and December 31, 2006.

Materials and Methods

Description of Data

Microbiological results from the FDA DICCP for the period January 1, 2004 through December 31, 2006, representing results from analysis of 17,324 total cheese samples, were obtained through a Freedom of Information Act request by the Cheese Choice Coalition and subsequently shared with us. Data analysis proceeded using methods specified by D'Amico and Donnelly (2011). These authors limited their analysis to the pathogens (described below) and did not conduct an analysis of generic (non-toxigenic) *E. coli* levels in tested cheese samples.

FDA collected cheeses according to the 1998 DICCP procedures (D'Amico and Donnelly, 2011). The FDA established the following priority for sample collection: (i) soft cheese, (ii) hard cheese, and (iii) cheese products. Domestic and imported cheese samples were categorized and tested for the presence of *L. monocytogenes*, *Salmonella*,

E. coli, Enterotoxigenic E. coli (ETEC) (only if *E. coli* levels exceeded 10,000 MPN/g), *Enterohemorrhagic E. coli* (EHEC), and *S. aureus* (U.S. FDA, 1998). Imports such as cheese wheels, loaves, or bricks that weighed 2.27 kg (5 lbs) or greater had two intact units from the same lot collected for further analysis. Retail units that weighed 454 g (1 lb) to not greater than 2 kg (5 lbs) had ten units (subsamples) collected from the same lot. When retail units weighed less than 454 g (1 lb), a collection was acquired that was equivalent to one sample (i.e. 10 subsamples). For domestic cheeses, the same policies applied for retail units that weighed between 454 g (1 lb) to not greater than 2 kg (5 lbs) and less than 454 g (1 lb). No policy for domestic cheeses that weighed equivalent to or greater than 2.27 kg (5 lbs) was mentioned.

According to the 1998 DICCP, once composites of subsamples were removed for *L. monocytogenes* and *Salmonella* spp. assays, portions were taken for further testing of non-toxigenic *E. coli*, ETEC, EHEC, and *S. aureus*.

E. coli enumeration proceeded as outlined in the FDA Bacteriological Analytical Manual (BAM) (Chap. 4) (U.S. FDA-BAM, 2002; U.S. FDA-BAM, 2010; U.S. FDA CFSAN, 2016). The FDA describes the MPN method as a statistically based, multi-step assay consisting of completed phases. Ten-fold serial dilutions of five sub samples (50 grams each) were blended with 450 milliliters of buffer. Samples were then inoculated into Lauryl tryptose (LST) or lactose- based broth media-containing test tubes and confirmed by presence or absence of gas and acid production via fermenting lactose after incubation at 35°C ± 0.5°C for 48 ± 3 h. Gas positive tubes had aliquots removed for sub-culture into EC broth for *E. coli*, incubation for 24 ± 2 h at 44.5°C, and examination for gas production. If gas production was negative, the cultures were re-incubated and

examined again at 48 ± 2 h. to re-confirm gas production. Once positive gas samples were established, aliquots of those samples were cultured on selective agar and incubated for 18-24 h at 35°C ± 0.5°C to isolate colonies for further confirmation with biochemical tests for the identification of *E. coli*. Identifying any 1 of the 5 colonies as *E. coli* was sufficient to confirm an *E. coli* tube as positive. These results were entered into a statistical table to estimate the number of *E. coli* present in the sample.

If non-toxigenic *E. coli* levels exceeded 10,000 MPN/g the subsample was tested for the presence of Enterotoxigenic *E. coli* (ETEC) as described by the FDA Bacteriological Analytical Manual (BAM) (Chap. 4A), Diarrheagenic *E. coli* (U.S. FDA, 1995; U.S. FDA 1998). The BAM Manual (Chapter 4A) indicates that ETEC levels are enumerated to assess the potential hazard of the contaminated food product. The 1998 DICCP used methods such as the Y-1 adrenal cell assays or commercial reverse passive latex agglutination assay and ELISA to detect the LT toxin. ST toxin was detected by ELISA or by infant mouse assay. Both LT and ST genes have also been sequenced and can be detected using PCR and gene probe assays.

The detection of EHEC was completed as described in Chapter 4 of the FDA BAM manual: "Isolation Methods for Enterohemorrhagic *E. coli* (EHEC)" under the 1998 DICCP (U.S. FDA, 1995; U.S. FDA 1998). Isolation was conducted using Tellurite-Cefixime-Sorbital MacConkey (TC SMAC) agar. These methods have been subsequently revised in FDA's 2017 Bacteriological Analytical Manual (BAM) (Chap. 4A), Diarrheagenic *E. coli* (U.S. FDA, 2017). Current methods describe screening for O157:H7 by using either the SmartCycler II or LightCycler® 2.0 platforms. These methods use modified Buffered Peptone Water with pyruvate (mBPWp) that contain anti-

microbial reagents to suppress indigenous microbial growth, which allow O157:H7 cells to grow (including other STEC). Both of these methods are capable of detecting <1 CFU/g in foods.

Data from FDA was provided to us in hard copy format, including 391 pages presenting data on all cheese samples tested between January 1, 2004 and December 31, 2006. Results for all samples tested were compiled and entered into a Microsoft® Office Excel (© 2010 Microsoft Corporation) document for further analysis.

For comparative purposes, the levels expressed during data analysis of *E. coli* results were normalized since the FDA employs MPN/g, while the EU determines CFU/g. For purposes of our analysis, levels were reported on a CFU/g basis. For all data where numbers were expressed as <(x) (i.e. <5), x (i.e. 5) was used for the numerical data when analyzed, and therefore results are more conservative. Samples where "no *E. coli"* was found and had no corresponding *E. coli* results were included in the data analysis as <3 *E. coli*/g. Samples that could not be included in the analysis due to incomplete data (as a result of the printing of information contained in text boxes) were identified and categorized. Samples that did not mention generic *E. coli* testing and did not have any *E. coli* results were categorized as not tested ("NT"). Cheese samples that were described, as being tested for generic *E. coli* but had no accompanying results were identified as "no data" ("ND"). Samples that had incomplete descriptions of generic *E. coli* tests were categorized as "unknown".

Data Analysis

We analyzed compliance of sample results with program criteria and their correlation with established guidance. Levels of 10,000/g, 1,000/g, 100/g and 10/g were established in our analysis to conform with microbiological criteria established for non-toxigenic *E. coli* from the 1998 Domestic and Imported Cheese Compliance Program (10,000 MPN/g), EU and 2015 Health Canada criteria (1000 CFU/g), 2009 CPG (100 MPN/g), and 2015 CPG regulations (10 MPN/g), respectively. Frequency tables were constructed to determine the statistical significance of cheeses that exceeded and complied with *E. coli* standards. EU and Health Canada criteria were included in this study because the majority of imported cheeses were from Europe and the FDA has previously collaborated with Health Canada to conduct a joint risk assessment regarding incidence of listeriosis due to soft-ripened cheese consumption (Health Canada and U.S. FDA, 2015).

IBM SPSS Statistics Version 24 Software was used to complete cross-tabulation tables for further analysis (Lowry, 2016). P-values were determined by computing "the significance of the difference between two independent proportions" using the Vassar Stats website. P-values were computed using p_a (sample A)-p_b (sample B) to provide a p-value that determined statistical significance based on a computed z-value. Sample A and sample B were calculated using p=k/n, where "k" is samples positive or exceeding a specified level out of "n", total samples tested for that *E. coli* level. Associations were found to be statistically significant when $p < 0.05$.

Correlation Coefficients

Correlation coefficients were determined between *E. coli* levels and presence of either *L. monocytogenes* or *Salmonella*, and between *S. aureus* levels exceeding 10,000 CFU/g

and the presence of *L. monocytogenes* and *Salmonella.* EHEC was not included because of the low incidence of this pathogen (3 positive/3,360 samples tested). These analyses were done to determine the efficacy of non-toxigenic *E. coli* as a food safety indicator.

Results

The FDA tested a total of 3,435 cheese samples for generic (typical) *E. coli* under the Domestic and Imported Cheese Compliance Program regulatory standards between January 1, 2004 and December 31, 2006. Data was available for our analysis from 3,007 of those samples and analyzed herein.

Out of 3,007 cheese samples tested, 76% (2,300/3,007) of cheeses contained *E. coli* levels that exceeded 10 *E coli*/g. Only 24% (707/3,007) of samples complied with the 2015 *E. coli* microbiological criteria (Table 2). Out of the total samples tested, 205 (6.8%) samples were tested for *E. coli* but no results were specified (ND), 157 (5.2%) were categorized as not tested (NT) for *E. coli*, and six (0.2%) samples were declared as "unknown". When compared to the 2009/2010 CPG standard of 100 *E. coli*/g, 68% (2,047/3,3007) of cheeses had levels that exceeded the standard and only 32% (960/3,007) of cheese samples complied. Of cheese samples tested, only 7.7% (232/3,007) of cheeses exceeded the EU Thermized and Health Canada 2015 criteria of 1,000 *E. coli*/g, while 92% (2,775/3,007) of cheeses complied with this standard. When compared to the 1998 Compliance Program criteria, only 5.7% (170/3,007) of cheese samples exceeded 10,000 *E. coli*/g, while 94% (2,837/3,007) of samples were able to comply with this standard.

Out of the cheese samples tested, our analysis identified that the top seven cheese

types achieving compliance during 2004-2006 were: (i) Cheese, Mexican Style, Soft, (ii) Cheese (Standardized) N.E.C., (iii) Cheese and Cheese Products, N.E.C. (Not Elsewhere Mentioned), (iv) Cheese, Hard, and equally, (v) Cheese, Cheddar, (vi) Cheese, Pasteurized, Process (Standardized), and (vii) Soft Ripened Cheese, Cow's Milk (Table 3).

The 1998 Compliance Program also required testing of cheese samples for the presence of *L. monocytogenes* and *Salmonella* and results obtained between January 1, 2004 and December 31, 2006 were analyzed for correlation with *E. coli* levels. Two-tailed chi-square tests revealed that cheese samples with higher *E. coli* levels (1,000/g and 10,000/g) showed stronger statistically significant correlations with cheese samples that tested positive for *L. monocytogenes* and *Salmonella*, while cheese samples containing *E. coli* levels of 100/g and 10/g did not (Table 4 and 6). Out of the 73 cheese samples that were both tested for both *L. monocytogenes* and had *E. coli* levels that exceeded 10,000 MPN/g, 26% (19 samples) tested positive for *L. monocytogenes.* Meanwhile, out of 2,764 cheese samples that were tested for *L. monocytogenes* and had *E. coli* levels of <10,000 MPN/g, only 0.7% (19) cheese samples tested positive for *L monocytogenes*. The difference between these two proportions determined that their association was statistically significant with a p-value <0.0002. Correlations were also determined between the EU Thermized/Health Canada 2015 *E. coli* standard of 1,000 *E. coli*/g and presence of *L. monocytogenes* in cheeses. The proportional difference between 18 cheese samples (0.7%) that tested positive for *L. monocytogenes* out of 2,717 cheese samples that comply with 1,000 *E. coli*/g standard and 20 cheese samples (17%) that tested positive for *L. monocytogenes* out of the total 120 cheeses that exceeded 1,000 *E.*

coli/g established statistical significance with a p-value of 0.0002. No statistically significant correlation was shown for samples that tested positive for *L. monocytogenes* and met the 2009/2010 CPG standard of 100 *E. coli*/g and 2015 CPG standard of 10 *E. coli*/g (p-values of 0.3778 and 0.2644 respectively). These correlations were determined after establishing the difference between the proportions of 9 cheese samples (1.0%) that tested positive for *L. monocytogenes* out of a total of 910 cheeses tested that comply with the 100 *E. coli*/g criterion and 29 cheese samples (1.5%) that tested positive for *L. monocytogenes* out of a total of 1,927 cheeses that exceed the 2009/2010 CPG standard. The lack of statistical significance for cheese samples meeting the 2015 *E. coli* criteria of 10/g was determined by establishing the difference between the proportions of 7 cheeses samples (1.0%) that tested positive for *L. monocytogenes* out of a total of 696 cheeses tested that comply with the 10 *E. coli*/g criterion and 31 cheese samples (1.4%) that tested positive for *L. monocytogenes* out of a total of 2,141 cheeses that exceed the 2015 CPG standard. Out of 2,837 total samples that were tested for *L. monocytogenes* and non-toxigenic *E. coli,* one sample (0.03%) was indeterminate (ND) and two samples (0.07%) were categorized as "not tested" (NT).

The majority of cheeses that tested positive for *L. monocytogenes* were Mexican-style soft cheeses, however soft, semisoft, soft cheese made from cow's milk, soft-ripened cheese made from cow's milk and goat's milk, cheese and cheese products, N.E.C., semisoft, blue, non-standardized products, and Monterey cheese were also included (Table 5). Out of the 41 cheese samples that tested positive for *L. monocytogenes* and could be accounted for, Mexican-style cheeses comprised 20 of these samples. Of these 20 samples tested, one sample contained <300 *E. coli*/g, one sample

contained *E. coli* levels of 1,500->110,000 *E. coli*/g, 11 samples contained 24,000->110,000 *E. coli*/g, and 7 samples contained 460,000->1,100,000 *E. coli*/g. One sample of soft, semisoft cheese made from cow's milk contained <3 *E. coli*/g, while two semisoft cheese samples contained <300 *E. coli*/g. Soft-ripened cheese made from cow's milk comprised 7 of the cheese samples. Out of these 7 *L. monocytogenes* positive cheese samples, 3 samples contained <3 *E. coli*/g, 2 samples contained <300 *E. coli*/g, one sample contained 3.6-7.4 *E. coli*/g, and another sample contained <30-4,600 *E. coli*/g. One soft-ripened cheese sample made from goat's milk contained <300 *E. coli*/g. Meanwhile, one cheese sample tested that was categorized under cheese and cheese products, N.E.C. also had <300 *E. coli*/g. The two blue cheese samples tested contained <300 *E. coli*/g and <3 *E. coli*/g, one Monterey cheese sample tested contained <3-3.6 *E. coli*/g, and one non-standardized product contained 43-11,000 *E. coli*/g. *L. monocytogenes* was detected in two samples that were not tested (NT) for *E. coli* and one sample that had no specified results (ND). The one sample that had no specified results and one of the samples not tested were categorized under sheep, N.E.C. The other sample not tested was categorized under the cheese type, cheese products, non-standardized, N.E.C, in addition to a sample that contained 43-11,000 *E. coli*/g.

The same methods used to determine the correlation and statistical significance of associations between *L. monocytogenes* positive samples and *E. coli* levels were applied to samples that tested positive for *Salmonella* (Table 6). Of the 106 cheese samples that exceeded the 1998 Compliance program *E. coli* standard of 10,000 *E. coli*/g and were tested for *Salmonella*, 31% (33 samples) tested positive in comparison to the 6 (0.2%) positive samples out of 2,777 that complied with the *E. coli* standard. The difference in

these proportions determined that the association was highly statistically significant with a p-value <0.0002. Correlations were also determined between the EU Thermized/Health Canada 2015 *E. coli* standard of 1,000 *E. coli*/g and presence of *Salmonella* in cheeses. The proportional difference between 6 cheese samples (0.2%) that tested positive for *Salmonella* out of 2,728 cheese samples that comply with 1,000 *E. coli*/g standard and 33 cheese samples (21%) that tested positive for *Salmonella* out of the total 155 cheeses that exceeded 1,000 *E. coli*/g showed a strong statistically significant association with a p-value of 0.0002. Overall, stronger statistical associations were found between high *E. coli* levels and *Salmonella* presence when compared to samples that achieved the lower *E. coli* levels of 100 *E. coli*/g (p-value of 0.0255) and 10 *E. coli*/g (p-value could not be determined due to a low sample size where numerators must be equal to five or greater).

The lack of statistically correlated significance was determined after establishing the difference between the proportions of 6 cheeses samples (0.6%) that tested positive for *Salmonella* out of a total of 921 cheeses tested that comply with the 100 *E. coli*/g criterion and 33 cheese samples (1.7%) that tested positive for *Salmonella* out of a total of 1,962 cheeses that exceed the 2009/2010 CPG standard. Statistical significance could not be determined between the 2015 *E. coli* criteria of 10 *E. coli*/g and samples that were found positive for *Salmonella* because the number of samples that complied with the 2015 CPG standard and tested positive for *Salmonella* (1/693) was below the numerator requirement of five needed for computational analysis. Out of 2,883 total samples that were tested for *Salmonella* and non-toxigenic *E. coli,* one sample (0.03%) was indeterminate (ND).

Similar to cheese types identified with presence of *L. monocytogenes*, Mexican-

style, soft cheeses were most prominently associated with presence of *Salmonella*, with other cheese types including standardized N.E.C., pasteurized process, cheese, hard, and soft-ripened cheese made from cow's milk (Table 7). Out of 40 total cheese samples that tested positive for *Salmonella* and were accounted for, Mexican-style cheeses comprised 25 of these samples. Of these 25 samples tested, one sample contained <30 *E. coli*/g, four samples contained 36-43,000 *E. coli*/g, 10 samples contained 93- >11,000 *E. coli*/g, two samples contained 4,600- 11,000 *E. coli*/g, two samples contained 11,000 *E. coli*/g, four samples contained 5,300- >110,000 *E. coli*/g, and two samples contained 46,000-110,000 *E. coli*/g. All nine samples of cheeses categorized as standardized, N.E.C. contained 2,400- >11,000 *E. coli*/g. Four cheese samples categorized under pasteurized process contained <3-23 *E. coli*/g. One soft-ripened cheese sample made from cow's milk contained <3 *E. coli/*g. One sample categorized under cheese, hard was tested positive for *Salmonella* and was tested for generic *E. coli* with no corresponding specified results.

Two-tailed chi-square tests also found statistically significant associations between cheese samples exceeding *S. aureus* levels of 10,000 CFU/g and the presence of *Salmonella* and *L. monocytogenes*, with p-values of <0.0002 (Table 8 and 9). To determine the association between the presence of *Salmonella* and *S. aureus* levels in cheese samples, differences between the proportions of five cheese samples (0.2%) that tested positive for *Salmonella* out of a total of 2,559 cheese samples that met the 1998 Compliance Program *S. aureus* standard of 10,000 CFU/g and 28 cheese samples (17%) out of a total of 163 samples that exceeded the 10,000 CFU/g criteria. Associations between cheese samples exceeding *S. aureus* levels of 10,000 CFU/g and presence of *L. monocytogenes* were determined by the difference between proportions of 18 cheese

samples (0.7%) that tested positive for *Salmonella* out of a total of 2,554 cheese samples that met the 1998 Compliance Program and 17 cheese samples (15%) out of a total of 115 samples that exceeded the 10,000 CFU/g criteria.

Cheese samples were also organized by cheese type and corresponding *E. coli* levels to determine cheese types most impacted by the 2015 Compliance Program regulatory standards (Table 10). All three years consecutively identified (i) Mexican-style soft cheeses, (ii) semisoft cheeses, (iii) soft ripened cheeses made with cow's milk, (iv) cheese made with goat's milk N.E.C., and (v) cheese made from sheep's milk N.E.C., as cheese types most affected by the 10 *E. coli*/g standard (Table 9). When combining all three consecutive years, 1, 1, 131, and 138 cheeses made from goat's milk N.E.C.; 70, 90, 176, and 205 Mexican-style soft cheeses; 10, 14, 268, and 284 semisoft cheeses; 6, 7, 165, and 174 cheese s made from sheep's milk N.E.C.; and 21, 24, 380, and 398 soft–ripened cheeses made from cow's milk exceeded the 10,000/g, 1,000/g, 100/g, and 10/g standards, respectively.

Discussion

A retrospective analysis of FDA's DICCP results was conducted to assess the impact of the FDA's 2015 Compliance Program non-toxigenic *E. coli* criteria on domestic and imported cheeses tested between January 1, 2004 and December 31, 2006. In order to promote cheese safety, establishment of science-based, prevention-oriented microbiological standards are necessary. Results of our analysis suggest that establishment of stringent non-toxigenic *E. coli* criteria had a limited impact on public health, as no significant correlations were found between low *E. coli* levels and presence

of *Salmonella* or *L. monocytogenes* in tested samples. However, statistically significant associations were found between FDA's target *S. aureus* levels and *Salmonella* or *Listeria*, confirming the appropriateness of EU food safety criteria that target *S. aureus* as the pathogen of concern in cheese.

The establishment of stringent non-toxigenic *E. coli* limits in raw milk cheese is having an adverse impact on cheeses produced domestically along with those being imported into the U.S. On September 8, 2014, the FDA issued a Constituent Update on the status of artisanal cheese in response to concerns that FDA was banning Roquefort or other cheeses (U.S. FDA, 2014). As stated by FDA, "Recent media reports have incorrectly indicated that the FDA is banning Roquefort and other cheeses. Earlier in 2014, nine producers of Roquefort, Tomme de Savoie, Morbier, and other cheeses tested above threshold levels set in 2010 for a particular type of bacteria called non-toxigenic *E. coli*. While these bacteria don't cause illness, their presence suggests that the cheese was produced in unsanitary conditions. The FDA has been working with the American Cheese Society (ACS) to learn more about artisanal cheeses and measures that cheesemakers take to ensure their products are safe. After hearing ACS' concerns about the test results, the FDA adjusted its criteria for taking regulatory action based on them. As a result, 95 percent of the cheese sampled tested below the level at which FDA would take regulatory action, and six of the nine cheese producers placed on Import Alert 12-10 for exceeding bacterial counts have been removed from that list and can resume sales and distribution in the U.S."

In its letter to the American Cheese Society dated October 30, 2014, in response to questions regarding how FDA established its *E. coli* microbiological criteria, FDA

writes “In deciding upon a final level for M, FDA considered ICMSF advice that, as a general hygiene indicator, “M” should represent clearly unacceptable conditions of hygiene. The scientific literature, international standards in use, and FDA’s own analytical results for non-toxigenic *E. coli* in cheese, led the agency to conclude that M at 100 MPN/g is consistently attainable and that exceeding this level in cheese is indicative of conditions meeting the adulteration standard of section 402(a)(4) of the FD&C Act” (U.S. FDA/HHS, 2014). The FDA further writes in its 10/30/2014 letter: “following issuance of the 1996 CPG, the domestic dairy industry shared its concerns with FDA regarding the permissible level of non-toxigenic *E. coli*. The concerns were that permitting up to 10,000 MPN/g of product 1) creates the appearance that the U.S. allows some domestically manufactured dairy products to be produced under insanitary conditions, and 2) poses an obstacle to exporting domestically manufactured dairy products, as export markets question why U.S. dairy products would be permitted to have such levels of non-toxigenic *E. coli*.” FDA guidance stands in contrast to guidance from ICMSF Book 8, published in 2011. Table 23.7 outlines end product testing criteria for cheeses. In cheeses made from pasteurized milk, *E coli* limits are established under a sampling plan where n=5, c=3, m=10 and $M=10^2$ (Table 1). Raw milk cheese is tested for *Staphylococcus aureus* only, consistent with EU recommended sampling criteria.

Based upon our retrospective analysis of the FDA’s 2004-2006 data, as *E. coli* criteria became more stringent, the number of cheeses that did not achieve compliance increased significantly, with 76% of cheese samples analyzed by the FDA between 2004-2006 exceededing 10 *E. coli*/g. For this reason, ICMSF and EU sampling plan guidelines (ICMSF, 1986; EU, 2005; ICMSF, 2011; CIFD, 2014) recommend the Confederation

Internationale des Fromagers Detaillants (CIFD) (2014) approach, which recognizes that even very good hygienic practices cannot guarantee absence of *E. coli* in raw milk prior to heat treatment. Therefore, CIFD does not propose *E. coli* standards for raw milk cheese, as generic *E. coli* is not considered a public health concern or a sanitary risk in France. However, ICMSF guidance has established a sampling plan targeting *E. coli* for cheeses made from heat-treated milk where n=5, c=3, m=10 and $M=10^2$ (ICMSF, 2011). FDA's application of these criteria to raw milk cheese is inconsistent with ICMSF guidance. Instead, ICMSF has established targets for *S. aureus* only in raw milk cheese where n=5, c=2, $m=10^4$ and $M=10^5$. Therefore, the statistical significance found between cheeses exceeding the *S. aureus* criteria of 10,000 CFU/g and presence of both *L. monocytogenes* and *Salmonella* implies that the 10,000 CFU/g criterion serves as an appropriate food safety indicator. This coincides with EU microbiological criteria and *Codex Alimentarius* for cheeses made from raw milk which target *S. aureus,* and not non-toxigenic *E. coli.*

Statistical significance between *E. coli* levels and presence of *L. monocytogenes* and *Salmonella* in cheese samples declined as *E. coli* standards became more stringent. These results are consistent with findings reported by Trmčič et al., (2016) who determined that 22% of raw milk cheese samples had detectable levels of *E. coli* (>10 CFU/g) but only 1.8% of cheese samples tested positive for *L. monocytogenes*. The FDA also released a Microbiological Sampling Assignment Summary Report (U.S. FDA CFSAN, 2016) on July 21, 2016 as part of a preventive sampling approach to eliminate contaminated foods from reaching consumers. The FDA analyzed 1,606 samples of raw milk cheeses that were aged for at least 60 days and were tested between 2014 and 2016.

The FDA found that 1,519 (95%) out of 1,606 total cheeses tested did not have violative levels of generic *E. coli* and only 13 (<1%) of these cheeses tested positive for *Salmonella*, *L. monocytogenes*, *E. coli O157:H7* and Shiga toxin-producing *E. coli* combined. Out of the cheeses tested, only 5.4% of samples were found to exceed *E. coli* levels established in the 3-class sampling plan proposed in 2015. Out of the 87 samples where violative *E. coli* levels were found, 18 samples were domestic and 69 samples were imported. These samples primarily consisted of semi-soft and hard cheeses, with the exception of three imported soft-ripened cheeses. It should be noted, however, that many of the cheeses in commerce between 2004 and 2006 were not in commerce between 2014 and 2016 due to their failure to comply with stringent U.S. *E. coli* criteria.

Out of the total number of samples tested, only one sample was found to have both violative levels of *E. coli* and presence of a pathogen. The FDA concluded that the presence of generic *E. coli* in the cheeses sampled did not correlate with presence of pathogens and was not useful in determining pathogen contamination in cheese. Similar findings were observed in our analysis of FDA's data from the Cheese and Cheese Product Compliance Program between January 1, 2004 and December 31, 2006. For example, a p-value could not be computed for samples that met the 2015 standard and tested positive for *Salmonella*, because only one cheese sample met that criteria, further demonstrating that low generic *E. coli* levels do not correlate with the presence of pathogens. The findings of this study are also consistent with outbreak data from Gould and colleagues (2014), who show that unpasteurized *queso fresco* (or other Mexican-style cheese) and *Salmonella*, and pasteurized *queso fresco* (or other Mexican-style cheese) and *L. monocytogenes*, are the most common cheese-pathogen pairs.

In our analysis, when observing associations between *E. coli* and *L. monocytogenes* and *Salmonella*, *E. coli* levels as low as <3 MPN/g were associated with cheese samples that tested positive for both pathogens. This level is below the 10 MPN/g lower standard of the sampling plan (n=5, c=3, m=10, M=100), meaning that these cheese samples would be accepted according to 2015 Compliance Program standards. This establishes that accepting these cheeses based on non-toxigenic *E. coli* levels ignores risk assessments that identify *L. monocytogenes* and *S. aureus* as appropriate science-based food safety indicators. As such, the value of *E. coli* testing is questioned and targeting specific pathogens of concern may be more appropriate to achieve food safety.

Comparisons with data from D'Amico and Donnelly (2011) further demonstrated the limitations of *E. coli* as a food safety indicator. While 76% of cheeses exceeded the 2015 Compliance Program *E. coli* criteria, only 339 samples (2.0%) out of a total of 17,324 tested samples contained pathogens (D'Amico and Donnelly, 2011). Also, out of 3,360 domestic and imported cheese samples analyzed, only 3 were positive for *E. coli* O157:H7. The low incidence of pathogenic *E. coli* found in cheese samples reaffirms the validity of ICMSF guidance in proposing no sampling plan for *E. coli* in cheese made from raw milk.

During 2004-2006, the number of cheese samples that exceeded 10,000 MPN/g and tested positive for pathogens declined as cheesemakers were making effort to comply with the 1998 Compliance Program. These results are also consistent with data presented by D'Amico and Donnelly (2011) and the 2016 FDA Summary Report, who demonstrate

that production of pathogen free cheese by cheesemakers was and continues to be achieved.

As of February 9, 2016, the FDA has paused its *E. coli* sampling of cheeses, but continues with testing for *L. monocytogenes*, *E. coli* O157:H7, Shiga toxin-producing *E. coli* (STEC), *Salmonella*, and *S. aureus* (U.S. FDA CFSAN, 2016). These findings establish that *E. coli* is not considered an appropriate indicator to determine presence of pathogens in cheese.

While, the FDA supports the use of *E. coli* to indicate presence of filth (Edberg et al., 2000; U.S. FDA, 2002; Stevens et al., 2003; Paruch and Maehlum, 2012), certain lineages of *E. coli* are not associated with fecal sources and yet are undifferentiated (Walk et al., 2009; Luo et al., 2011; Oh et al., 2012). Studies have challenged the use of coliforms and *E. coli* as indicator organisms (Stevens et al., 2003; Wu et al, 2011), suggesting that mutation of housekeeping genes can occur outside of the mammal gastrointestinal tract (Walk et al., 2009). Therefore, using generic *E. coli* as a hygiene indicator for raw milk cheeses is debatable (IDF, 2016). Testing for *E. coli* may have merit in determining whether the cheesemaking process is allowing *E. coli* levels to decline, or in cheeses made from heat-treated milk, whether or not post-process contamination has occurred. According to the International Dairy Federation (IDF), *E. coli* will grow during the cheesemaking process and will reach the highest levels within the first couple weeks of ripening. This suggests that the most opportune time to test for *E. coli* is after the first one to two weeks of ripening. Once this time surpasses, *E. coli* levels decline and testing the finished cheese product has little value (IDF, 2016). IDF also states that during the cheesemaking process, *E. coli* levels will increase by 10-fold,

as a result of bacteria concentration within the curd as whey is removed. Therefore, hygiene standards for cheeses should target *E. coli* levels that are 10 times greater than the *E. coli* standards for fluid milk (IDF, 2016). This reasoning is derived from the understanding that hard cheeses create a competitive environment due to the presence of other microbiological communities, nutrient availability, water activity, and pH that inhibit *E. coli* from surviving (IDF, 2016).

Despite ICMSF and EU guidance based upon scientific risk assessment, the FDA cited two studies to support its establishment of non-toxigenic *E. coli* criteria, as evidence that the microbiological limits of 10 MPN/g will not cause barriers for domestic or imported cheeses. Brooks et al. (2012) tested 41 hard, semi-soft, and soft cheeses that were obtained nation-wide for analysis of non-toxigenic *E. coli*. Out of the 41 cheeses samples tested, only two met or exceeded 10 MPN/g. The FDA also cited a study that observed the presence of pathogens and non-toxigenic *E. coli* in 351 farmhouse cheeses (O'Brien et al, 2009). Results showed that 79% of those cheeses tested for non-toxigenic *E. coli* fell below 10 MPN/g. Given the small number of cheese samples analyzed in these two studies, it is unclear whether these results extend to broader cheese categories.

The FDA established its non-toxigenic *E. coli* standards in the 2015 Compliance Program Guidelines in order to align its programs with the goals of FSMA, which mandates a risk-informed and preventive approach to food safety (U.S. FDA, 2013). However, FSMA also mandates that we harmonize our food safety regulations with those of our global trading partners and the 2015 Domestic and Imported Cheese Compliance Program requirements are inconsistent with microbiological criteria utilized by the EU.

Health Canada has also established less stringent *E. coli* criteria and sampling

plan for raw milk cheeses (n=5, c=2, m=10^2 and M=10^3) (Health Canada, 2015; Health Canada and U.S. FDA, 2015). This 3-class sampling plan specifies that cheese samples are considered adulterated if non-toxigenic *E. coli* levels of greater than 100 CFU/g and less than 1,000 CFU/g are found in 2 or more of 5 subsamples, or if one subsample exceeds 1,000 CFU/g.

This retrospective analysis allows us to probe the question of the impact of stringent *E. coli* standards on cheese commerce, particularly U.S. artisan and European PDO and AOC cheeses legally produced from raw milk. If 76% of cheese samples tested between 2004 and 2006 had *E. coli* levels greater than 10 MPN/g, there can be no question that these standards are having an adverse impact on cheese commerce, favoring those products made from heat treated milk, which can easily achieve this standard. Results from this analysis of the FDA's 2004-2006 data are similar to findings from the FDA's 2014-2016 data report that specifies the lack of association between generic *E. coli* levels and presence of pathogens in cheese samples. The FDA's report also affirms the adverse impacts on cheese commerce if cheeses are to comply with such stringent microbiological criteria.

Conclusion

Results of our analysis confirm the limited food safety value of FDA's use of stringent microbiological criteria for *E. coli* in domestic and imported cheeses. These stringent criteria are having a major impact on cheese commerce without affording food safety benefits. Since most cheeses produced by the U.S. domestic dairy industry are produced from heat-treated milk, employment of EU microbiological criteria where n=5, c=2,

m=10^2, and M=10^3 for cheeses made from heat treated milk only, would: achieve harmonization of EU and U.S. standards for cheese; address the concerns expressed by the U.S. domestic dairy industry; and address the concerns expressed by the American Cheese Society who represent the U.S. artisan cheese makers who use raw milk in the production of their cheeses. EU microbiological criteria for coagulase positive *S. aureus* where n=5, c=2, m=10^4 and M=10^5 for cheeses made from raw milk would also achieve these outcomes.

Limitations

While this data analysis provides insight into the impact of FDA's 2015 non-toxigenic *E. coli* criteria on U.S. domestic and imported cheeses, there are limitations. The data set is organized in such a way that the sampling plan for non-toxigenic *E. coli* specified by the 2015 Compliance Program (n=5, c=3, m=10, M=100) could not be taken into consideration when completing the analysis. Also, our analysis could not determine which samples tested were raw milk or pasteurized cheeses; therefore, all samples were represented in the analysis as pasteurized and raw milk cheeses.

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Table 1: REGULATION (EU) No. 1441/2007, microbiological criteria

Food	Bacteria	Sampling Plan		Limits (CFU/g)		Method
		n	c	m	M	
Cheese with thermal treatment	*E. coli* (hygienic index)	5	2	10^2	10^3	ISO 16649-1 ó 2
	Coagulase positive Staphylococci	5	2	10^2	10^3	EN/ISO 6888-2
Raw milk cheese	Coagulase positive Staphylococci	5	2	10^4	10^5	EN/ISO 6888-2
	E. coli	N/A		N/A		

Table 2: Number of cheese samples analyzed by FDA under the Domestic and Imported Cheese Compliance Program between January 1, 2004 and December 31, 2006 exceeding non-toxigenic *E. coli* levels

Guidelines	*E. coli* Levels (*E. coli*/g)	No. samples exceeding criteria (%)	No. samples complying with criteria (%)
2015 CPG	>10	2,300/3,007 (76)	
	<10		707/3,007 (24)
2009/2010 CPG	>100	2,047/3,007 (68)	
	<100		960/3,007 (32)
EU Thermized/Health Canada 2015	>1,000	232/3,007 (7.7)	
	<1,000		2,775/3,007 (92)
1998 Compliance Program	>10,000	170/3,007 (5.7)**	
	<10,000		2,837/3,007 (94)

[a]Samples were tested for generic *E. coli* and no *E. coli* was found but results were not mentioned.

[b]Indeterminate results (No Data) stated that samples were tested for generic *E. coli*, but *E. coli* levels were not available due to missing data from hard copies.

[c]Samples that could not be determined as tested for generic *E. coli* were categorized as "unknown".

[d]Samples that had no information regarding *E. coli* testing or results were categorized as "not tested" (NT).

**FDA reported 292/3,345 (8.7%) of cheese samples exceeding 10,000 *E. coli*/g

Table 3: Number of cheese samples analyzed by FDA under the Domestic and Imported Cheese Compliance Program between January 1, 2004 and December 31, 2006 that met the <10 *E. coli*/g compliance criteria by cheese type

Cheese Type	FY 04	FY 05	FY 06	Total
Cheese (Standardized), N.E.C.	13	12	28	53
Cheese and Cheese Products, N.E.C.	25	20	33	78
Cheese Food, Cold Pack (Standardized)	-	1	1	2
Cheese Products, Non-Standardized. N.E.C.	5	4	10	19
Cheese Products Standardized, N.E.C.	6	3	1	10
Cheese, Asiago, Fresh, Medium Soft and Old	-	3	-	3
Cheese, Blue	4	6	5	15
Cheese, Brick	-	-	1	1
Cheese, Cheddar	14	11	9	34
Cheese, Cheese Pasteurized, Processed with Fruits, Vegetables, or Meats	-	-	-	0
Cheese, Colby	6	4	1	11
Cheese, Cold Pack, Club	1	-	-	1
Cheese, Cook, Koch	-	1	1	2
Cheese, Cream	4	6	2	12
Cheese, Cream with Other Foods	1	-	-	1
Cheese, Edam	1	-	-	1
Cheese, Goat, N.E.C.	9	6	6	21
Cheese, Gorgonzola	-	-	-	0
Cheese, Gouda	5	2	1	8
Cheese, Gruyere	1	-	-	1
Cheese, Hard	17	18	15	50
Cheese, Hard Grating	-	2	-	2
Cheese, Havarti	2	-	2	4
Cheese, Jack, High Moisture	-	1	-	1
Cheese, Limburger	-	-	-	0
Cheese, Mexican Style, Soft	45	15	19	79
Cheese, Monterey	4	2	2	8
Cheese, Muenster	2	3	2	7
Cheese, Natural, Smoked (Non-Standardized)	2	-	-	2
Cheese, Neufchatel	-	-	-	0
Cheese, Neufchatel, Pasteurized with other Foods (Standardized)	1	-	-	1
Cheese, Parmesan	2	-	1	3
Cheese, Pasteurized, Blended (Standardized)	-	-	-	0

Cheese, Pasteurized, Process (Standardized)	21	9	4	34
Cheese, Pizza	3	-	1	4
Cheese, Provolone	2	4	2	8
Cheese, Reggiano	6	-	-	6
Cheese, Ricotta	5	3	5	13
Cheese, Romano	1	4	3	8
Cheese, Roquefort	-	-	1	1
Cheese, Semisoft	5	10	5	20
Cheese, Semisoft, Part Skim	8	2	2	12
Cheese, Sheep, N.E.C.	6	13	6	25
Cheese, Skim for Manufacturing	-	-	-	0
Cheese, Soaked Curd	2	-	-	2
Cheese, Spiced (Standardized)	1	-	-	1
Cheese, Stilton	-	-	-	0
Cheese, Stirred Curd (Standardized)	-	1	1	2
Cheese, Swiss, Emmentaler	11	2	4	17
Cheese, Syrian	1	1	1	3
Cheese, Washed Curd (Standardized)	-	-	-	0
Cottage Cheese, (Not <4% Milk Fat)	8	2	-	10
Cottage Cheese, Dry Curd (<0.5% Milk Fat)	-	3	-	3
Cottage Cheese, Low Fat (0.5-2% Milk Fat)	4	1	-	5
Pasteurized Cheese Spread	2	-	-	2
Pasteurized Cheese Spread with Fruits, Vegetables, or Meats	-	-	-	0
Pasteurized Process Cheese Foods	4	1	-	5
Pasteurized Process Cheese Foods with Fruits, Vegetables, or Meats	-	-	-	0
Pasteurized Process Cheese Spread	3	-	-	3
Pasteurized Process Cheese Spread with Fruits, Vegetables, or Meats	2	1	-	3
Queso Crema	-	-	-	0
Soft Ripened Cheese, Cows Milk	10	12	12	34
Soft Ripened Cheese, Goat's Milk	4	5	9	18
Soft Ripened Cheese, Mixture of Animal Milk	-	2	-	2
Soft Ripened Cheese, Sheep's Milk	-	1	-	1
Soft, Semi-Soft Cheese, Cow's Milk	12	12	9	33

Table 4: Incidence of *Listeria monocytogenes* in cheese samples analyzed by FDA under the Domestic and Imported Cheese Compliance Program between January 1, 2004 and December 31, 2006 and association with non-toxigenic *E. coli* levels

Guidelines	*E. coli* Levels (*E. coli*/g)	No. of *L.m.* positives/ No. total samples tested (%)	p-value
2015 CPG	<10	7/696 (1.0)	0.3778
	>10	31/2141 (1.4)	
2009 CPG	<100	9/910 (1.0)	0.2644
	>100	29/1927 (1.5)	
EU Thermized/Health Canada 2015	<1,000	18/2717 (0.7)	<0.0002*
	>1,000	20/120 (17)	
1998 Compliance Program	<10,000	19/2764 (0.7)	<0.0002*
	>10,000	19/73 (26)	
Indeterminate	No Data (ND)	1/2837 (0.03)[a]	N/A
Not Tested (NT)	NT	2/2837 (0.07)[b]	N/A

[a]Indeterminate results (No Data) showed that samples were tested for non-toxigenic *E. coli*, but *E. coli* levels were not available due to missing data from hard copies.
[b]Samples that had no information regarding *E. coli* testing or results were categorized as "not tested" (NT).
*Statistical significance was determined by computing the difference between two independent proportions ($p < 0.001$)

Table 5: *L. monocytogenes* positive cheese samples as tested by FDA under the Domestic and Imported Cheese Compliance Program between January 1, 2004 and December 31, 2006 and corresponding non-toxigenic *E. coli* levels by cheese type

Cheese Type	*E. coli* Levels (*E. coli*/g)
Blue	<300
	<3
Cheese and Cheese Products, N.E.C.	<300
Cheese Products, Non-Standardized, N.E.C.	NT*
	43-11,000
Mexican-Style, Soft	<300
	<300
	1,500- >110,000
	24,000- >110,000
	24,000- >110,000
	24,000- >110,000
	24,000- >110,000
	24,000- >110,000
	24,000- >110,000
	24,000- >110,000
	24,000- >110,000
	24,000- >110,000
	24,000- >110,000
	460,000- >1,100,000
	460,000- >1,100,000
	460,000- >1,100,000
	460,000- >1,100,000
	460,000- >1,100,000
	460,000- >1,100,000
	460,000- >1,100,000
Monterey	<3- 3.6
Non-Standardized Products	43- 11,000
Semisoft	<300
	<300
Sheep, N.E.C.	NT[a]
	ND[b]
Soft, Semisoft, Cow	<3
Soft-Ripened, Cow	<3
	<3
	<3
	3.6- 7.4
	<30- 4,600
	<300
	<300
Soft-Ripened, Goat	<30

[a]Sample was not tested (NT) for generic *E. coli*.
[b]Sample was tested for generic *E. coli* but no results were specified.
*Sample was not reported by the FDA

Table 6: Incidence of *Salmonella* in cheese samples analyzed under the Domestic and Imported Cheese Compliance Program between January 1, 2004 and December 31, 2006 and association with non-toxigenic *E. coli* levels

Guidelines	*E. coli* Levels (*E. coli*/g)	No. positives No. total samples tested (%)	p-value
2015 CPG	<10	1/693 (0.1)	Cannot be determined[b]
	>10	38/2190 (1.7)	
2009 CPG	<100	6/921 (0.6)	0.0255
	>100	33/1962 (1.7)	
EU Thermized/Health Canada 2015	<1,000	6/2728 (0.2)	<0.0002*
	>1,000	33/155 (21)	
1998 Compliance Program	<10,000	6/2777 (0.2)	<0.0002*
	>10,000	33/106 (31)	
Indeterminate	No Data (ND)	1/2883 (0.03)[a]	N/A

[a]Indeterminate results (No Data) showed that samples were tested for non-toxigenic *E. coli*, but *E. coli* levels were not available due to missing data from hard copies.
[b]Statistical significance could not be determined due to a low sample size where numerators must be equal to five or greater.
*Statistical significance was determined by computing the difference between two independent proportions ($p < 0.001$).

Table 7: *Salmonella* positive cheese samples as tested by FDA under the Domestic and Imported Cheese Compliance Program between January 1, 2004 and December 31, 2006 and corresponding non-toxigenic *E. coli* levels by cheese type

Cheese Type	*E. coli* Levels (*E. coli*/g)
Cheese, Hard	ND[a]
Mexican-style soft	<30
	>11,000
	>11,000
	36- 43,000
	36- 43,000
	36- 43,000
	36- 43,000
	4,600- 11,000
	4,600- 11,000
	46,000- 110,000
	46,000- 110,000
	5,300- >110,000
	5,300- >110,000
	5,300- >110,000
	5,300- >110,000
	93- >11,000
	93- >11,000
	93- >11,000
	93- >11,000
	93- >11,000
	93- >11,000
	93- >11,000
	93- >11,000
	93- >11,000
	93- >11,000
Pasteurized Process	<3- 23
	<3- 23
	<3- 23
	<3- 23
Soft-Ripened, Cow	<3
Standardized, N.E.C.	2,400- >11,000
	2,400- >11,000
	2,400- >11,000
	2,400- >11,000
	2,400- >11,000
	2,400- >11,000
	2,400- >11,000
	2,400- >11,000
	2,400- >11,000

[a]Sample was tested for generic *E. coli* but no results were specified.

Table 8: Incidence of *Salmonella* in cheese samples analyzed associated with incidence of *Staphylococcus aureus* under the Domestic and Imported Cheese Compliance Program between January 1, 2004 and December 31, 2006

Guidelines	*S. aureus* Levels (CFU/g)	No. positives No. total samples tested (%)	p-value
1998 Compliance Program	<10,000	5/2559 (0.2)	<0.0002*
	>10,000	28/163 (17)	

*Statistical significance was determined by computing the difference between two independent proportions ($p < 0.001$).

Table 9: Incidence of *Listeria monocytogenes* in cheese samples analyzed associated with incidence of *Staphylococcus aureus* under the Domestic and Imported Cheese Compliance Program between January 1, 2004 and December 31, 2006

Guidelines	*S. aureus* Levels (CFU/g)	No. positives No. total samples tested (%)	p-value
1998 Compliance Program	<10,000	18/2554 (0.7)	<0.0002*
	>10,000	17/115 (15)	

*Statistical significance was determined by computing the difference between two independent proportions ($p < 0.001$).

Table 10: Number of cheese samples analyzed under the Domestic and Imported Cheese Compliance Program between January 1, 2004 and December 31, 2006 exceeding non-toxigenic *E. coli* levels by cheese type

Cheese Type	>10,000/g**	>10,000/g	>1,000/g	>100/g	>10/g
Cheese (Standardized), N.E.C.	23*	20	26	100	107
Cheese and Cheese Products, N.E.C.	39*	23	44	104	123
Cheese Food, Cold Pack (Standardized)	-	-	-	8	8
Cheese Products, Non-Standardized. N.E.C.	9*	1	2	43	60
Cheese Products Standardized, N.E.C.	-	-	-	1	1
Cheese, Asiago, Fresh, Medium Soft and Old	1*	-	-	1	1
Cheese, Blue	5*	-	-	51	59
Cheese, Brick	1*	-	-	7	7
Cheese, Cheddar	14*	4	5	42	75
Cheese, Cheese Pasteurized, Processed with Fruits, Vegetables, or Meats	-	-	-	1	1
Cheese, Colby	1*	-	-	18	18
Cheese, Cold Pack, Club	-	-	-	1	1
Cheese, Cook, Koch	1*	-	-	-	-
Cheese, Cream	-	-	-	11	15
Cheese, Cream with Other Foods (Standardized)	-	-	-	6	7
Cheese, Edam	-	-	-	13	13
Cheese, Goat, N.E.C.	2*	1	1	131	138
Cheese, Gorgonzola	-	-	-	13	13
Cheese, Gouda	10*	5	5	45	50
Cheese, Gruyere	-	-	-	4	5
Cheese, Hard	11*	-	3	82	97
Cheese, Hard Grating	1*	-	-	4	4
Cheese, Havarti	-	-	-	29	31
Cheese, Jack, High Moisture	1*	-	-	3	6
Cheese, Limburger	-	-	-	1	1
Cheese, Mexican Style, Soft	100*	70	90	176	205
Cheese, Monterey	2*	-	-	17	26
Cheese, Muenster	2*	-	-	23	24
Cheese, Natural, Smoked (Non-Standardized)	-	-	-	2	2
Cheese, Neufchatel	-	-	-	-	-
Cheese, Neufchatel, Pasteurized with other Foods (Standardized)	-	-	-	-	-
Cheese, Parmesan	-	-	-	10	10
Cheese, Pasteurized, Blended (Standardized)	-	-	-	1	1
Cheese, Pasteurized, Process (Standardized)	10*	2	3	29	35
Cheese, Pizza	-	-	-	3	4

Cheese, Provolone	1*	-	-	16	18
Cheese, Reggiano	-	-	-	19	19
Cheese, Ricotta	5	5	5	15	17
Cheese, Romano	1*	-	-	13	13
Cheese, Roquefort	-	-	-	10	10
Cheese, Semisoft	16*	10	14	268	284
Cheese, Semisoft, Part Skim	-	-	-	20	26
Cheese, Sheep, N.E.C.	8*	6	7	165	174
Cheese, Skim for Manufacturing	-	-	-	5	5
Cheese, Soaked Curd	-	-	-	3	4
Cheese, Spiced (Standardized)	-	-	-	2	3
Cheese, Stilton	-	-	-	7	7
Cheese, Stirred Curd (Standardized)	-	-	-	7	7
Cheese, Swiss, Emmentaler	3*	-	-	25	26
Cheese, Syrian	-	-	-	-	-
Cheese, Washed Curd (Standardized)	-	-	-	4	5
Cottage Cheese, (Not <4% Milk Fat)	-	-	-	5	6
Cottage Cheese, Dry Curd (<0.5% Milk Fat)	-	-	-	3	6
Cottage Cheese, Low Fat (0.5-2% Milk Fat)	-	-	-	5	8
Pasteurized Cheese Spread	-	-	-	5	6
Pasteurized Cheese Spread with Fruits, Vegetables, or Meats	-	-	-	5	5
Pasteurized Process Cheese Foods	-	-	-	-	-
Pasteurized Process Cheese Foods with Fruits, Vegetables, or Meats	-	-	-	1	1
Pasteurized Process Cheese Spread	-	-	-	3	3
Pasteurized Process Cheese Spread with Fruits, Vegetables, or Meats	-	-	-	-	-
Queso Crema	-	-	-	1	1
Soft Ripened Cheese, Cows Milk	15*	21	24	380	398
Soft Ripened Cheese, Goat's Milk	2*	-	1	45	57
Soft Ripened Cheese, Mixture of Animal Milk	1	1	1	2	5
Soft Ripened Cheese, Sheep's Milk	2*	1	1	9	9
Soft, Semi-Soft Cheese, Cow's Milk	2*	-	-	24	29

**Cheese samples with (typical) non-toxigenic *E. coli* levels that exceeded 10,000 MPN/g according to FDA results.
*Difference between discrepant results reported by the FDA and researchers would need to be taken into consideration for all other *E. coli* levels.

References

American Cheese Society (ACS). (2014). Position statement on the safety of aging cheese on wood; issued in response to the recent food and drug administration (FDA) statement on the use of wooden shelves for cheese aging. http://campaign.r20.constantcontact.com/render?ca=7bbd724d-fd30-4d4f-ac09-3817c2f59aa6&c=198439d0-1d97-11e3-9012-d4ae527557ea&ch=19e65700-1d97-11e3-901a-d4ae527557ea. Accessed 8.2.2015.

Brooks, J.C., Martinez B., Stratton, J., Bianchini, A., Krokstrom, R., & Hutkins, R. (2012). Survey of raw milk cheeses for microbiological quality and prevalence of foodborne pathogens. Food Microbiology, 31, 154-158.

Confederation Internationale des Fromagers Detaillants (CIFD). (2014). Note de Synthèse Problématique du critère *E. coli* sur les fromages au lait cru imposé par la FDA (English translation). http://iccheesemongers.com/wp-content/uploads/synth%c3%a8se-english-translation-vweb.pdf. Accessed 4.17.17.

Correll, W.A. (2014). Letter to American Cheese Society (ACS) with an Update on What Has Been Done with Respect to Non-Toxigenic *Escherichia coli* (*E. coli*) in Raw Milk Cheese, Public Health Service, Food and Drug Administration, College Park, MD.

D'Amico, D.J., Druart, M.J. and Donnelly, C.W. (2014). Comparing the Behavior of Multidrug-Resistant and Pansusceptible *Salmonella* during the Production and Aging of a Gouda Cheese Manufactured from Raw Milk. *J Food Prot*. 77(6), pp. 903–913.

D'Amico, D.J., and Donnelly C.W. (2011). FDA's Domestic and Imported Cheese Compliance Program Results: January 1, 2004- December 31, 2006. *J Food Prot* Trends. 31(4), 216-226.

D'Amico, D.J., Druart, M.J. and Donnelly, C.W. (2008). 60-day aging requirement does not ensure safety of surface-mold-ripened soft cheeses manufactured from raw or pasteurized milk when *Listeria monocytogenes* is introduced as a post-processing contaminant. *J Food Prot*. 71(8), pp.1563–1571.

Department of Health and Human Services. U.S Food and Drug Administration (FDA). (2015). Compliance Program for Domestic and Imported Cheese and Cheese Products. Chapter 3: Foodborne Biological Hazards. (Update of 1998 Food Compliance Program for Domestic and Imported Cheese and Cheese Products. Chapter 3: Foodborne Biological Hazards.). http://www.fda.gov/downloads/Food/ComplianceEnforcement/FoodCompliancePrograms/UCM456592.pdf. Accessed 11.5.2015.

Department of Health and Human Services. U.S. Food and Drug Administration (FDA). (2006). Code of Federal Regulations, chap. 1, subchapter B, title 21, part 133. Cheeses and related cheese products. Section 133.182: soft ripened cheeses. Available at: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr133.182. Accessed 9.22.17.

Department of Health and Human Services. U.S. Food and Drug Administration (FDA). (2002). Compliance Policy Guide: ‘‘Filth from Insects, Rodents, and Other Pests in Food’’; Availability. Federal Register, Vol. 67, No. 231 / Monday, December 2, 2002 / Notices. https://www.gpo.gov/fdsys/pkg/FR-2002-12-02/pdf/02-30403.pdf

Department of Health and Human Services. U.S Food and Drug Administration (FDA). (1998). Compliance Program for Domestic and Imported Cheese and Cheese Products. Chapter 3: Foodborne Biological Hazards. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/ ComplianceEnforcement/UCM071510.htm. Accessed 8.20.15.

Edberg, S. C., E. W. Rice, R. J. Karlin, and M. J. Allen. (2000). *Escherichia coli*: The best biological drinking water indicator for public health protection. *Symp. Ser. Soc. Appl. Microbiol*. (29):106S–116S.

European Commission. (2015). Food Law General Principles. http://ec.europa.eu/food/safety/general_food_law/principles/index_en.htm. Accessed 8.10.2015.

European Union (EU). (2005). Commission regulation (EC No 2073/2005 of 15 November 2005 on microbial criteria for foodstuffs. Official Journal of the European Union. L338, 1-26. http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32005R2073&from=en. Accessed 4.13.17

Evans J.W., (1968). The General Agreement on Tariffs and Trade. International Organization, 22(01), 72.

FAO. (1996). Corporate Document Depository. http://www.fao.org/docrep/meeting/005/w2198e/w2198e0a.htm. Accessed 8.2.2015.

Froman, M. (2014). Office of the United States Trade Representative. 2014 Report on Sanitary and Phytosanitary Measures. (Revised from 1994), 1-116. https://ustr.gov/sites/default/files/FINAL-2014-SPS-Report-Compiled.pdf. Accessed 4.16.17.

Gay, M., Cerf, O. and Davey, K.R., 1996. Significance of pre-incubation temperature and inoculum concentration on subsequent growth of *Listeria monocytogenes* at 14°C. *J App Microbiol.* 81(4), pp.433–438.

Genigeorgis C., M. Carniciu, D. Dutulescu, and T.B. Farver. 1991. Growth and survival of *Listeria monocytogenes* in market cheeses stored at 4 to 30°C. *J. Food Prot*. 54:662-668.

Gould, Hannah L., Mungai, Elisabeth, Behravesh, Casey B. (2014). Outbreaks Attributed to Cheese: Differences Between Outbreaks Caused by Unpasteurized and Pasteurized Dairy Products, United States, 1998–2011. J Foodborne Pathogens and Disease (11). doi: 10.1089/fpd.2013.1650

Hargrove, R.E., McDonough F.E., and. Maltingly M.A. (1969). Factors affecting survival of *Salmonella* in cheddar cheese and colby cheese. *J. Milk Food Technol.* 32:480-484.

Health Canada. (2015). Canadian Dairy Information Centre; National Dairy Code. http://www.dairyinfo.gc.ca/index_e.php?s1=dr-rl&s2=canada&s3=ndc-cnpl&s4=05-2005. Accessed 11.24.2016.

Health Canada and U.S. Food and Drug Administration (FDA) (2015). Joint FDA/Health Canada Quantitative Assessment of the Risk of Listeriosis from Soft-Ripened Cheese Consumption in the United States and Canada. https://www.fda.gov/downloads/Food/FoodScienceResearch/RiskSafetyAssessment/UCM429419.pdf

Institute for Trade and Commercial Diplomacy (ITCD). (1999) Codex Alimentarius Commision. http://www.commercialdiplomacy.org/ma_projects/ma_dairy_hygiene2.htm.htm#_ftn27. Accessed 8.8.2015.

International Commission of Microbiological Specifications of Foods (ICMSF). (2011). Microorganisms in Foods Book 8: Use of Data for Assessing Process Control and Product Acceptance. http://www.icmsf.org/publications/books.html. Accessed 6.22.2015.

International Commission of Microbiological Specifications of Foods (ICMSF). (1986). Microorganisms in Foods Book 2: Use of Data for Assessing Process Control and Product Acceptance. http://www.icmsf.org/publications/books.html. Accessed 6.22.2015.

International Dairy Federation (IDF). (2016). *Escherichia coli* as indicator in cheese processing. http://www.fil-idf.org/wp-content/uploads/2016/12/Escherichia-coli-as-indicators-in-cheese-processing.pdf. Accessed 2.8.2017.

Le Loir Y., Baron F., and Gautier M. (2003). *Staphylococcus aureus* and food poisoning. *Genet. Mol. Res*. 2 (1): 63-76.

Larson, A.E., Johnson, E.A. and Nelson, J.H. (1999). Survival of *Listeria monocytogenes* in Commercial Cheese Brines. *J Dairy Sci.* 82(9), pp.1860–1868. Available at: http://dx.doi.org/10.3168/jds.S0022-0302(99)75419-6.

Leyer, G.J. and Johnson, E. A. (1993). Acid adaptation induces cross-protection against environmental stresses in *Salmonella typhimurium*. *Appl. Environ. Microbiol.* 59(6), pp.1842–1847.

Leyer, G. J., and E.A. Johnson. (1992). Acid adaptation promotes survival of *Salmonella* spp. in cheese. *Appl. Environ. Microbiol*. 58:2075-2080.

Lowry R. (2016). VassarStats: Website for Statistical Computation. http://vassarstats.net/. Accessed 10.3.2016.

Luo, C., Walk, S. T., Gordon, D. M., Feldgarden, M., Tiedje, J. M., and Konstantinidis, K. T. (2011). Genome sequencing of environmental *Escherichia coli* expands understanding of the ecology and speciation of the model bacterial species. Proceedings of the National Academy of Sciences, 108(17), 7200-7205.

Meyrand, A., Boutrand-Loei S., Ray-Gueniot S., Mazuy C., Gaspard, C.E., Jaubert G., Perrin G., Lapeyre C., Vernozy-Rozand C. 1998. (1998). Growth and enterotoxin production of *Staphylococcus aureus* during the manufacture and ripening of Camembert-type cheeses from raw goats' milk. *J App Microbiol*. 85(3), pp.537–544.

O'Brien M., Hunt K., McSweeney S., and Jordan K. (2009). Occurrence of foodborne pathogens in Irish Farmhouse Cheese. *J Food Microbiology*, 26(8), 910–914.

Oh, S., S. Buddenborg, D. R. Yoder-Himes, J. M. Tiedje, and K.T. Konstantinidis. (2012). Genomic diversity of Escherichia isolates from diverse habitats. *PLoS One* 7:e47005. http://dx.doi.org/10.1371/journal.pone.0047005.

Olsen, AR., Gecan, JS., Ziobro, GC., Bryce, JR. (2001). Regulatory Action Criteria for Filth and Other Extraneous Materials V. Strategy for Evaluating Hazardous and Nonhazardous Filth, Regulatory Toxicology and Pharmacology, 33(3): 363-392. ISSN 0273-2300. https://doi.org/10.1006/rtph.2001.1472.

Park, H.S., Marth E.H., Goepfert J.M., Olson N.E., (1972). The fate of *Salmonella typhimurium* in the manufacture and ripening of low-acid Cheddar cheese. *Journal of Milk and Food Technology*. 33(7), pp.280–284.

Paruch, A. M., and T. Maehlum. (2012). Specific features of *Escherichia coli* that

distinguish it from coliform and thermotolerant coliform bacteria and define it as the most accurate indicator of faecal contamination in the environment. Ecol. Indic. 23:140–142. http://dx.doi.org/10.1016/j.ecolind.2012.03.026.

Smith J., Buchanan R., Palumbo S., (1983). Effect of food environment on staphylococcal enterotoxin synthesis: A review. *J Food Protection*, 46(6), pp.545–555. Available at: http://www.researchgate.net/publication/257308319_Effect_of_Food_Environment_on_Staphylococcal_Enterotoxin_Synthesis_A_Review/file/60b7d524e1d6ab2cd3.pdf.

Stevens, M., N. Ashbolt, and D. Cunliffe. (2003). Review of Coliforms as Microbial Indicators of Drinking Water Quality. Recommendations to Change the Use of Coliforms as Microbial Indicators of Drinking Water Quality. Australian Government, National Health and Medical Research Council. Biotext Pty Ltd., Canberra, Australia.

Trmčić, A., Chauhan, K., Kent, D. J., Ralyea, R. D., Martin, N. H., Boor, K. J., and Wiedmann, M. (2016). Coliform detection in cheese is associated with specific cheese characteristics, but no association was found with pathogen detection. Journal of Dairy Science, 99(8), 6105-6120.

U.S. Food and Drug Administration (FDA). (2017) (revised from 1995). Bacteriological Analytical Manual (BAM): Diarrheagenic *Escherichia coli*. Bacteriological Analytical Manual. Chapter 4A. https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070080.htm. Accessed 9.29.2017.

U.S. Food and Drug Administration (FDA). (2016). Center for Food Safety and Applied Nutrition (CFSAN). FY 2014 – 2016 Microbiological Sampling Assignment Summary report: raw milk cheese aged 60 days. Available at: http://www.fda.gov/Food/NewsEvents/ConstituentUpdates/default.htm?source=govdelivery&utm_medium=email&utm_source=govdelivery. Accessed 7.26.2016.

U.S. Food and Drug Administration (FDA) Center for Food Safety and Applied Nutrition CFSAN) (2014). CFSAN Constituent Update. Clarification on Using Wood Shelving in Artisanal Cheesemaking. http://wayback.archive-it.org/7993/20171114120232/https:/www.fda.gov/Food/NewsEvents/ConstituentUpdates/ucm400808.htm. Accessed December 3, 2018.

U.S. Food and Drug Administration (FDA) Department of Health and Human Services (HHS) (2014). FDA's Response Letter to the American Cheese Society. http://www.cheesesociety.org/wp-content/uploads/2014/11/Response-to-American-Cheese-Society.pdf?utm_source=Copy+of+Member+Update-CPG+for+Non-Toxigenic+E.+coli&utm_campaign=Member+Update+11-5-14&utm_medium=email. Accessed 8.2.2015.

U.S. Food and Drug Administration (FDA). (2014). FDA Update on the Status of Artisanal Cheese. http://www.fda.gov/Food/NewsEvents/ucm413309.htm. Accessed 8.26.2015.

U.S. Food and Drug Administration (FDA). (2013). FDA's International Food Safety Capacity Building Plan. Food Safety Modernization Act Section 305. http://www.fda.gov/downloads/Food/GuidanceRegulation/UCM341440.pdf. Accessed 11.29.16.

U.S. Food and Drug Administration (FDA). (2010). Bacteriological Analytical Manual (BAM) Appendix 2: Most Probable Number from Serial Dilutions. https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm109656.htm. Accessed 4.13.17.

U.S. Food and Drug Administration (FDA). Center for Food Safety and Applied Nutrition (CFSAN) (2010). Compliance Policy Guide Sec. 527.300 Dairy Products-Microbial Contaminants and Alkaline Phosphatase Activity. 75 FR 80826. http://origin.www.gpo.gov/fdsys/pkg/FR-2010-12-12/pdf/2010-32232.pdf. Accessed 11.17.14.

U.S. Food and Drug Administration (FDA). (2009). Center for Food Safety and Applied Nutrition (CFSAN). Draft Compliance Policy Guide Sec. 527.300 Dairy Products-Microbial Contaminants and Alkaline Phosphatase Activity. 74 FR 62795. http://edocket.access.gpo.gov/2009/pdf/E9-28756.pdf. Accessed 11.9.14.

U.S. Food and Drug Administration (FDA). (2002). Bacteriological Analytical Manual (BAM): Enumeration of *Escherichia coli* and the Coliform Bacteria. Bacteriological Analytical Manual. Chapter 4. http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm064948.htm Accessed 6.22.2015.

U.S. Food and Drug Administration (FDA). (1999). USA Delegation Report for the Codex committee on Food Hygiene drafting Group Meeting for the Code of Hygienic Practice for Milk and Milk Products," 22-25- March, 1999. P. 2. Available from www.fsis.usda.gov:80/OA/codex/rep_brussesl.htm.

U.S. Food and Drug Administration (FDA). (1995). Bacteriological Analytical Manual (BAM): Diarrheagenic *Escherichia coli*. Bacteriological Analytical Manual. Chapter 4A; 8th Edition.

Walk, Seth T., Elizabeth W. Alm, David M. Gordon, Jeffrey L. Ram, Gary A. Toranzos, James M. Tiedje, and Thomas S. Whittam. (2009). Cryptic lineages of the genus Escherichia. *J App and Env Microbiol*. 75, no. 20: 6534–44. doi:10.1128/AEM.01262-09.

World Trade Organization (WTO). (1998). General Agreement on Tariffs and Trade. https://www.wto.org/english/res_e/booksp_e/agrmntseries2_gatt_e.pdf. Accessed 4.15.17

Wu, J., S. C. Long, D. Das, and S. M. Dorner. (2011). Are microbial indicators and pathogens correlated? A statistical analysis of 40 years of research. J. Water Health 9:265–278. http://dx.doi.org/10.2166/wh.2011.117

CHAPTER 3: Comparison of Survival of Generic *E. coli* and *Listeria* spp. in Dairy Compost- versus Poultry Litter Compost-Amended Soils in the Northeastern U.S. and Impact on Harvested Edible Crops (Radish)

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Running Head: Survival of Generic *E. coli* and *Listeria* spp. Populations

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Abstract

Application of FSMA-compliant BSAAO to soils for production of fresh produce is expected to result in reduced risk of pathogen contamination on the harvested produce when other stipulations in the Produce Safety rule are also implemented. However, meteorological conditions, geographic location, application methods, soil type, and bacterial populations can influence the presence of pathogenic bacteria, or their indicators (e.g., generic *E. coli*) and potential produce contamination. Replicated field plots ($2m^2$, *n* = 24) of loamy (L) or sandy (S) soils were tilled and amended with dairy compost (DC), poultry litter compost (PL), or no compost (NC) over two different field seasons. These plots were inoculated with a three-strain cocktail of rifampicin-resistant *E. coli* (r*E.coli*) at a rate of 8.7 log CFU/m^2. Colony count and most probable number (MPN) methods were used to determine persistence of r*E.coli* in these plots through 104 days post-inoculation (dpi). Detection of indigenous *Listeria* spp. were also examined in all plots. Higher r*E. coli* populations were observed in PLC plots (-0.04 to 2.07 log MPN/gdw) in comparison to DC plots (-0.06 to -0.88 log MPN/gdw) and NC plots (-0.56 to -0.89 log MPN/gdw) during year 1. Similar trends were observed for year 2, where at 102 dpi, inoculated r*E. coli* survived at higher population levels in PP plots (2.44 to 2.84 log MPN/gdw) and PLC plots (below detectable levels, i.e., -2.52 log MPN/gdw) in comparison to DC plots (-0.52 to 0.87 log MPN/gdw) and NC plots (-0.85 log MPN/gdw). Levels of r*E. coli* and native *E. coli* after rainfall events were independent of soil type. *Listeria* spp. were found in NC plots, but not in PL or DC. Radish data demonstrates that PL treatment (0.342-2.79 log MPN/radish sample) promoted the

greatest level of r*E.coli* translocation and survival when compared to DC (undetectable to 1.41 log MPN/radish sample) and NC (undetectable to 0.785 log MPN/radish sample) treatments. Results are consistent with those from studies conducted in other regions of the US that show that poultry litter-based BSAAO support greater numbers and longer periods of persistence in field soils of r*E. coli* than dairy-based BSAAO and can have an impact on edible crops grown in BSAAO amended soils.

Introduction

The Centers for Disease Control and Prevention (CDC) examined attribution of domestically acquired foodborne illnesses, hospitalizations, and deaths in the United States to specific commodities using outbreak data, and reported that produce accounted for 46 % of foodborne illnesses (Painter et al., 2013). The leading pathogen/produce combinations responsible for outbreaks in the U.S. are *Escherichia coli* associated with leafy greens, followed by *Salmonella* spp. and tomatoes, and *Salmonella* spp. associated with leafy greens (Anderson et al., 2011). *Listeria monocytogenes* is also a well-recognized food-borne pathogen that causes produce-related outbreaks of listeriosis, with an associated 30% mortality rate in immunocompromised populations (Locatelli et al., 2013). *L. monocytogenes* is widely distributed in decomposing plant material and manure (Hutchison et al, 2002; Nightingale et al., 2004; Park et al., 2012; Santorum et al., 2012).

Since the early 1970's, a considerable increase in the consumption of fresh produce has been observed in the U.S. (Harris et al., 2003) presumably due to the promotion of fruits and vegetables as part of a healthy diet. This increase in consumption of produce and better surveillance may be contributing to an increase in *E. coli* O157:H7 associated outbreaks. The economic burden in the U. S. alone due to foodborne illnesses attributed to *L. monocytogenes*, non-typhoidal *Salmonella* spp., and *E. coli* O157:H7 is approximately $2.0 billion, $4.4 billion, and $607 million, respectively (Park et al., 2012; Scharff, 2012). The industry costs are derived from

expenses on recalled product, sampling and testing, and preventive action to minimize contamination (Park et al., 2012). Demand for bagged spinach decreased by 43% during the year after *E. coli* O157:H7 was implicated in a large spinach outbreak in 2006 (Park et al., 2012). The financial and public health impacts warrant action to reduce pathogen contamination of produce.

President Obama signed the FDA Food Safety Modernization Act (FSMA) into law on January 4, 2011 and rules accompanying FSMA will be fully implemented by September 17, 2018 (U.S. FDA, 2011). With prevention being a primary focus of FSMA, it is now mandatory for all qualifying facilities to create and implement a written hazard analysis and risk-based preventive controls food safety plan. This plan must evaluate the hazards that could affect the safety of the food, specify what preventive controls will be put in place to minimize the hazards, describe how the controls will be monitored, maintaining records and allowing producers to determine frequency of implementation based on a risk-based approach that is consistent with its hazard analysis, and specify subsequent corrective actions. FSMA also requires implementation of minimum standards for the safe production and harvest of produce based on naturally occurring hazards. This takes into consideration biological soil amendments, hygiene, packaging, temperatures, animals in the immediate area, and water quality (U.S. FDA, 2011).

Biological soil amendments of animal origin (BSAAO) are materials including manure or other non-fecal byproducts such as cattle manure, poultry litter, swine slurry, or horse manure (U.S. FDA 21 CFR 112). The FDA Supplemental Proposed Rule for "Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption" has recognized that while (BSAAO) play an important role in providing

nutrients to improve soil and produce quality, they are also a potential source of microbial pathogen contamination (Sharma and Reynells, 2016). Pathogens of concern found in BSAAO include Enterohemorrhagic *E. coli* (EHEC), *Salmonella*, *Campylobacter jejuni*, *Cryptosporidium parvum* and *L. monocytogenes* (Harris et al., 2003; Sharma and Reynnells, 2016).

The survival of pathogens in manure-amended soils has been investigated previously (Harris et al., 2012; Islam et al., 2004; Jiang et al., 2002; Sharma and Reynnells, 2016; Natvig et al., 2002; Reynnells et al., 2014; Yao et al., 2015). However, most studies applied large population levels (10^8/g) of pathogens or indicator organisms, which may not represent conditions encountered by growers (Jiang et al., 2002). Brochier et al. examined the impact of compost- and manure amended-soils on survival of *Enterococcus* and *Clostridium perfringens* applied at levels of 1,000 CFU/g and 100 MPN/g, respectively (Brochier et al., 2012). Previous studies have also attempted to establish generic *E. coli* as an indicator for presence of pathogen contamination in soils and water sources intended to come into contact with produce (Cooley et al., 2007; Natvig et al., 2002). Survival of *S. enterica* serovar Typhimurium (4 to 5 log CFU/g) in bovine manure that was amended into silty clay loam (SCL) or loamy sand (LS) plots, died at similar rates (from 4.8 log CFU/g to 1.68 CFU/g) and was still detected 17 weeks after application. Generic *E. coli* levels fell from 1.29 log CFU/g to below detectable levels nine weeks later, when radishes, arugula, and carrots were planted. Generic *E. coli* is a useful indicator organism for evaluating risk of vegetable contamination with BSAAO, because *E coli* levels also exceed those of *S. enterica* serovar Typhimurium. Conflicting results were shown for water from Salinas Valley, which were analyzed for

coliforms and generic *E. coli* to determine the total maximum daily load (TMDL). There was a correlation between the incidence of *E. coli* O157:H7 and generic *E. coli* levels found in watershed samples when all data was pooled, but no significant correlation with individual water sample sites due to a low incidence of *E. coli* O157:H7 (Cooley et al. 2007). Given that *E. coli* O157:H7 was undetected in many samples with high generic *E. coli* levels, led to the conclusion that generic *E. coli* is a poor indicator of *E. coli* O157:H7 presence in water (Cooley et al., 2007).

E. coli O157:H7 persisted for 154 to 217 days in soils amended with poultry and bovine manure composts, which resulting in detection on lettuce and parsley for up to 77 and 177 days, respectively, after seedlings were planted in Maryland (Islam et al., 2004). Very little difference was observed in *E. coli* O157:H7 persistence based on compost type alone. In Vermont, *E. coli* had a 3-3.5 log reduction between days 0 and 56, in loamy and sandy soil, respectively, amended with dairy manure (Lekkas et al., 2016). Poultry litter-amended soils contained larger populations of generic *E. coli* and attenuated *E. coli* O157:H7 (2.84 to 2.88 log CFU/g [dry weight]) compared to dairy manure-amended (0.29 to 0.32 log CFU/gdw) or unamended (0.25 to 0.28 log CFU/gdw) soils (Sharma et al. 2016).

This extended survival of generic *E. coli* and *E. coli* O157:H7 in BSAAO may be attributed to higher nutrient (N:P) availability in poultry litter amendments. Nitrogen is a strong driver of *E. coli* survival (Franz et al., 2008) and in fresh manure, 60-80% of N is typically in an organic form (i.e., urea and protein) (Kelleher et al., 2002). Therefore, using manure and manure compost as soil amendments may allow translocation of *E. coli* O157:H7 to edible fruits and vegetables (Hirneisen, et al., 2012; Markland et al., 2013;

Patel et al., 2010; Sharma et al., 2016). The FDA recommends application of FSMA-compliant compost to soils instead of raw manure due to the reduced risk of pathogen contamination on the harvested produce when other stipulations in the Produce Safety rule are also implemented (U.S. FDA, 2018a). The concern associated with use of raw manure as a BSAAO is that domesticated animals tend to be reservoirs for pathogens (Sharma et al. 2016). Therefore, composting of BSAAO is a method that uses thermal inactivation as a means to eliminate pathogens by meeting mesophilic (ambient temperature to 40°C) and thermophilic (from 55 to 65°C) phase conditions (Singh et al., 2010). USDA National Organic Program standards require that when raw animal manure is applied to soil, an organic crop cannot be harvested for 90 days (if the edible portion does not have direct contact with the soil), or for 120 days for organic crops having direct soil contact (USDA, 2014). In cases where manure is composted according to NOP standards, there is no harvest restriction. NOP standards require compost to be held at temperatures of 55°C for at least 3 days, with a 45 day curing interval, before soil application (Islam et al., 2004; Sharma and Reynnells, 2016; Reynnells et al., 2014; Sharma et al., 2016).

Microbial standards that set limits on detectable amounts of bacteria (including *L. monocytogenes*, *Salmonella* spp., fecal coliforms, and *E. coli* 0157:H7) have been established for processes used to treat biological soil amendments, including manure (U.S. FDA, 2018a). Stabilized compost must be applied in a manner that minimizes the potential for contact with produce during and after application. The produce rule specifies that biological amendments that undergo a physical (thermal), chemical, or combined process must achieve the microbial standards under the Proposed Produce Rule

§§ 112.54(b) and 112.55(b) subparts, where it is specified that biosolids must contain <1000 most probable number (MPN)/g fecal coliforms for BSAAO that are treated and no *L. monocytogenes* may be detected in any 5 gram (or milliliter for a liquid) analytical sample *Salmonella* spp. cannot be detected above 3/MPN per 4 grams of total solids dry weight, and *E. coli* O157:H7 cannot be detected above 0.3 MPN per one gram analytical portion (U.S. FDA, 2018a). This rule specifies that compost can achieve standards by implementing (i) static composting in an oxygenated environment that targets 131°F (55°C) for a total of three days, followed by curing with proper insulation or, (ii) turned composting in an aerobic environment that targets 131°F (55°C) for 15 days throughout five turnings, followed by curing and proper insulation (U.S. FDA, 2013).

Composting can become less effective when materials are infrequently turned or when nutrient composition, pH, or moisture content are inadequate to meet the microbial activity needed to achieve proper heating parameters of the composted material (Singh et al. 2010). Achieving these standards is essential as the C:N ratio and moisture content all contribute to the regrowth of pathogens in finished compost (Reynnells et al., 2014), presenting a subsequent risk for produce contamination when these compost-amendments are intended for growing and harvesting edible crops. Sharma and Reynnells (2016) supported this by demonstrating that sterilized compost inoculated with *Salmonella* spp. provided enough nutrients to support growth when compared to non-sterilized composts. Cutler et al. (2018) also demonstrated similar trends with *E. coli*, where sterilized composts continued to support *E. coli* survival and persistence. Therefore, many environmental factors, including water activity, pH, aeration (turning) parameter, compost recipe, and characteristics of the growth medium need to be considered when

composting (Cebrián, et al., 2017). Other biotic factors to consider is a potential growth phase within the compost (Sharma and Reynnells, 2016). Temperatures that do not completely inactivate cells will allow cross-protection against other hurdling factors that may present themselves at a later time, such as pH (Cebrián et al., 2017). This is important to further investigate as bacteria can resuscitate growth if given the optimal growing parameters (Sharma and Reynnells, 2016).The type of soil that may be mixed with the compost also impacts survival and persistence of pathogens over time. Sandy soils tend to hold less moisture when compared to loamy soils (Fremaux, et al., 2008; Fremaux, et al., 2008b; Locatelli et al., 2013; Sharma et al., 2016).

The FDA is conducting a risk assessment on predicted risk of human illness associated with produce consumption from growing areas amended with untreated BSAAO that are potentially contaminated with enteric pathogens (*E. coli* O157:H7 or *Salmonella*), to evaluate the impact of different agricultural/ecological conditions and interventions that include use of a time interval or intervals between application of untreated BSAAO and harvest of edible crops (U.S. FDA, n.d.). This study was conducted to inform the risk assessment by (i) evaluating survival of non-pathogenic *E. coli* and indigenous *Listeria* spp. in tilled plots with dairy and poultry composts in the northeastern U.S. to determine FSMA-compliance when applying BSAAO to soils according to stipulations in the Produce Safety rule, (ii) determine taxonomy of microbial communities in compost-amended soil and non-compost-amended soil treatments, (iii) determine correlations between inoculated *E. coli* survival, days post inoculation (dpi), and taxonomy profiles in each treatment, and (iv) establish contamination trends onto

edible produce to determine potential for contamination from composted BSAAO amended soils.

Materials and Methods

Field Experimental Design

Two field trials were conducted using replicated field plots (2m x 1m, n=24) of loamy (L) (Field A) or sandy (S) (Field B) soils (Table 1). These field sites differed in soil composition. Field B was 80-82% sand, 5% clay, and 13-15% silt and Field A was 88-90% sand, 10% clay, and 0-2% silt (Culter et al., 2017). Treatments were arranged in a randomized complete block design. Year 1 and 2 treatments were replicated four and five times per treatment, respectively, for a total of n=24 and n=20 plots. Individual plots (2m x 1m) were separated by 1.5m (5ft) alley-ways to avoid border interference. These plots were tilled and amended with the following treatments: (i) no compost or rifampicin resistant *E. coli* (r*E. coli*) (negative control), (ii) no compost with r*E. coli* (positive control for *E. coli*), (iii) dairy compost and r*E. coli* (DC) and (iv) poultry litter compost and r*E. coli* (PL), where (v) a poultry pellet with r*E. coli* (PP) treatment was added for Trial 2. Methods for this study were adopted from those developed by Reynnells et al. (2014). For all treatments, composts were added first, then inoculated with r*E. coli*, and then tilled. Then, on the appropriate plots, r*E. coli* inoculum was dispensed using a backpack sprayer at a rate of 10^6 CFU/ml. The inoculum cocktail was dispensed and applied at the rate of 1-L per plot. The rototiller blades were sanitized with 75% ethanol

in-between tiling each treatment as specified by Cutler et al. (2018). Poultry compost and dairy composts were applied at a rate of 13.4 tons/acre (30,038.8 kg/ha) and 6.72 metric tons/acre (15,064.2 kg/ha), respectively (Cutler et al., 2018). Composts were obtained from research or commercial sources and were applied at the same rate (kg/plot) to ensure that methods could be comparable. Composts were spread evenly on plot surfaces.

Field Inoculum Preparation

Field inoculum preparation was completed as described by Lekkas et al. (2016). An inoculum cocktail was created with three strains of generic, non-pathogen, rifampicin resistant *E. coli* (*rE. coli*) (TVS 353, 354, 353), as noted in other field studies (Moyne et al., 2011; Sharma et al., 2016). The rifampicin (80 µg/ml) resistant r*E. coli* strains were used in this experiment to better differentiate them from indigenous *E. coli* populations (*gEc*). Tomás-Callejas et al.(2011) initially isolated the *rE. coli* strains, which were provided by the Environmental Microbial Food Safety Laboratory at the Beltsville Agriculture Research Center in Beltsville, MD 20705. TVS 355 has been isolated from lettuce production soil in the Salinas Valley area, TVS 353 was previously isolated from irrigation water, and TVS 354 has been isolated from Romaine lettuce surfaces (Gutiérrez-Rodríguez et al., 2012).

Preparation of Dairy Manure Extract

For this study, manure was collected from a university farm and was added to a 1:10 (100 grams manure: 900 mL ddH^2O) dilution of ddH^2O in a large (2L) Nalgene bucket after the manure was manually massaged to remove any large intact material. This solution

was stirred for 5 minutes. Sanitary cheesecloth was used to hand squeeze solids out of the extract to be used, collecting approximately ¾ the input H_2O volume. This extract was transferred to a clean carboy of equal ddH^2O to total 3 L of diluted extract (1:2) per carboy and sterilized.

Preparation of the Bacterial Inoculum

Three strains of r*E. coli* (TVS 353, 354, 355) were cultured separately in 100 ml TSB supplement with 80 mg/ml rifampicin (TSBR) at 37°C with agitation. Each 100 ml culture was added to a 3L carboy and shaken and incubated at 37°C, then stored at 4°C for no longer than 48 hours. Prior to field inoculation, counts were enumerated by plating 100 µl of the cultures onto TBXR with the appropriate dilutions to determine the amount needed from each culture to create the field inoculum. Depending on the population levels, an appropriate amount of each r*E. coli* strain from each culture was added to a carboy of sterilized water diluent before being transferred to a battery-powered backpack sprayer (Solo brand, 4-gallon). In total, 13-Liters of inoculum were made for field application. Prior to application, an aliquot of each diluent was removed and enumerated to determine population levels of the inoculum cocktail in each sprayer.

Media

All culturing media was made as described by Lekkas et al., (2016). The *rE. coli* strains were plated on Tryptone Bile X-Glucuronide (TBX) Chromogenic Agar which contained the chromagen 5-bromo-4-chloro-3-indolyl-β-D-glucuronide(Neogen Corp, Lansing MI), supplemented with 8 µg/L of rifampicin (TBXR) (Sigma-Aldrich, St.

Louis, MO). Native *gEc* was plated on TBX without rifampicin (TBX) using the same methods. Trypticase Soy Broth (Neogen Corp, Lansing MI) with rifampicin (TSBR) was used when determining most probable number (MPN) enumeration of *rE.coli.* Double strength (2x) and single strength (1x) of TSB medium were used when completing the MPN method. One ml of rifampicin was added to the single strength TSB and two ml of rifampicin were added to the double strength TSB once the broth solutions were cooled to room temperature.

ECC *E. coli* broth (Neogen Corp., Lansing MI) was also used to enumerate native *gEc* using the MPN method and no rifampicin was added. Buffered Peptone Water (BPW) was added to soil samples before all subsequent processing.

For the enrichment of soil samples for *Listeria* spp. detection, Buffered *Listeria* Enrichment Broth (BLEB) was used. Samples were incubated in non-selective media for 4h to enable injured or stressed cells to resuscitate, after which time acriflavin (3m/L), cyclohexamide (5ml/L), and nalidixic acid (8ml/L) were added. A secondary enrichment of MOPS-BLEB broth was also used. Once the MOPS-BLEB medium was cooled to room temperature, acriflavin (3m/L), cyclohexamide (5ml/L), and nalidixic acid (8ml/L) were added to the media prior to the soil sample being added. For further *Listeria* spp. isolation and identification, samples were plated onto ChromList™ (DRG International, Springfield, NJ) agar for further selectivity.

Selective Agents

Acriflavin and nalidixic acid stock solutions were prepared in a 0.5% (w/v) solution distilled and deionized water (ddH^2O). Cyclohexamide was prepared at a concentration

of 1% (w/v) in 40:60 ratio of ethanol to ddH^2O. Antibiotics were filtered with a 0.45 µg/ml syringe prior to subsequent addition to the appropriate medium. Rifampicin antibiotics were prepared by adding 8 g of rifampicin (powder form) to 100 ml of dimethyl sulfoxide (DMSO), stirred gently with slight heat, and then filtered through a 0.22 µm nylon filter.

Sampling Procedure

All plots had 3 core samples collected at random locations, which were transferred to a sterile WhirlPak™ bag. Each core sample was taken 15 cm deep below surface and sample stakes were applied to those areas to ensure that soil would not be re-sampled in that location. Samples were transported to the lab and hand massaged for 30 seconds to homogenize each subsample thoroughly. Thirty grams of each soil sample was suspended in 120 ml of buffered peptone water (BPW) to achieve a 1:5 dilution (w/w). BPW was made by dissolving 20 g of BPW powder into 1 L of ddH_2O and autoclaving the broth for 15 minutes at 121°C. These diluted samples were also manually massaged to create a homogenous mixture prior to further processing. Soil samples were collected on 0, 1, 3, 7, 14, 28, and 56 days post inoculation (dpi), followed by a monthly sampling thereafter.

Enumeration and Microbial Analysis of Samples

Enumeration and enrichment methods were similar to those described by Lekkas et al., (2016) and Sharma et al. (2016). Briefly, the control samples were processed for

enumeration and presence/absence using TBX to detect for indigenous non-rifampicin resistant *E. coli.* The remaining treatments were processed using TBXR to detect inoculated r*E. coli.* Plates were enumerated in duplicate with 100 µl of each sample (TSB/TSBR). Plates were incubated at 42°C for 24 h to determine number of colony forming units per gram (CFU/g). Once less than 20 colonies per plate were counted on both plates, samples were re-plated onto four plates using 250 µl per plate, plating a total of 1 ml. Samples were incubated at 42°C for 24 h. Once colony counts were below 20 CFU/ml per plate, *E. coli* counts were determined using the MPN method.

Most Probable Number (MPN) Method

An aliquot of one mL of sample was transferred into 1 mL of double strength (2x) TSBR or TSB for *rE. coli* or indigenous *E. coli* (*gEc)*, respectively in the first row of a 48 well block (8 rows x 6 columns x 5 mL wells), totaling a 2 mL mixture of sample and broth. Subsequent rows contained 1.8 ml of the appropriate broth, depending on the r*E. coli* to be enumerated. Serial dilutions were completed by aliquoting 200 µl of sample per well. Blocks were covered with a breathable Easy plate (VWR, Bridgeport, NJ) membrane and incubated at 42°C for 24 h. Each well was then plated on TBXR (*rE. coli*) or TBX (*gEc*) plates and incubated for 24 h at 42°C. MPN statistical computation was completed using a MPN calculator (VB6 version, www.i2workout.com/mcuriale/mpn/index.html), with a score of 8 (theoretical lower threshold of <0.11 MPN/g) and 4 (theoretical lower threshold of <0.23 MPN/g) for *rE. coli* and *gEc,* respectively, to determine viable cells.

Bag Enrichment

After r*E. coli* are below the detection threshold for MPN methods, bag enrichment was used to enrich r*E. coli* in non-selective BPW to verify presence or absence. Thirty grams of soil sample was suspended in 120 ml of BPW to create a 1:5 dilution and then massaged to achieve homogeneity. The sample was then placed into an incubator for 24 h at 42°C. These samples were then plated onto TBXR (*rE. coli*) or TBX (*gEc*) plates and incubated for another 24 h at 42°C. The radish sampling methods section below describes subsampling methodology when radish samples achieved bag enrichment.

***Listeria* spp. Identification**

Listeria presence in unamended soils and compost-amended soils was determined using enrichment methods (D'Amico & Donnelly, 2009). MOPS-BLEB dual enrichment was performed, then 100 µl was subsequently plated onto CHROMagar™ *Listeria* and incubated for 24 hours at 37°C. Confirmation of the *Listeria* spp. was completed using CHROMagar™ *Listeria* Identification agar to confirm *L. monocytogenes* from suspect colonies on CHROMagar™ *Listeria*. Isolated strains were subsequently assayed using the DuPont Qualicon BAX Q7 system (BAX PCR; DuPont Qualicon Wilmington, DE.) to detect presence of *Listeria* spp. Any presumptive *L. monocytogenes* positives that were confirmed through culturing methods were isolated and ribotyped using the Riboprinter® Microbial Characterization System by Hygiena (City).

Radish Sampling Methods

Radish seeds were planted by hand-broadcasting across all treatments. Throughout seedling establishment, radishes were randomly selected and aseptically removed from

plots and then transferred to a sterile Whirlpack™ bags (Natvig et al., 2002). Sterile scissors were used to remove the radish tops and a 30 to 55 g subsample was aseptically added to another sterile Whirlpack™ bag where 99 ml of Buffered Peptone-Water (Neogen Corporation, Acumedia) and hand massaged/shaken to avoid antimicrobial phytochemicals from impacting results. This method was used to enhance removal of microbes from the surface of the radishes. Once again, MPN methods were performed on each sample and when MPN results were negative, bag enrichment was completed to determine presence or absence of *rE. coli* growth. Weeds were also allowed to grow to better imitate plant rhizosphere dynamics on soil communities.

Soil Microclimate Monitoring

Soil temperatures were covariables that were recorded at every hour in each field at 10 cm depths throughout both field trials using the Campbell Scientific 10x dataloggers (Logan, UT). Soil temperatures and water potential were quantified using Thermister probes and Watermark™ probes, respectively. Percent moisture was determined gravimetrically (g water per g dry soil) for each soil sample that was collected.

Microbial Ecoenzymatic Activity

Enzyme activity was determined using hydrolase, amino-peptidase, and esterase activity to quantify microbial function and activity expressed as nmol h^{-1} gdw^{-1} (Cutler et al., 2018). Hydrolases (BG = β -glucosidase and NAG = β -1,4-Nacetylglucosaminidase) serve as indicators for hydrolysis of plant and fungal cell walls, respectively. L-leucine aminopeptidase (LAP) and phosphatase (PP) activity are indicators for degradation of

proteins, and phosphate, respectively (Moorhead et al., 2013). Enzymatic analyses demonstrates the allocation of energy produce by microbial communities to synthesize enzymes due to limited C, N or P, excluding energy from natural decay. BG to AP or BG to (NAG+LUC) ratios were plotted to compare microbial need for carbon, phosphorous, and nitrogen in soil through time (Sinsabaugh et al., 2012) (Table 2).

Bacterial and Fungal Community Compositions and Ordination

Genomic DNA was extracted using the MoBio PowerSoil DNA Isolation kit (Carlsbad, CA, USA) per manufacturer instructions using the methods described by Lauber et al., (2009). Samples were amplified at the University of Colorado Boulder using 515f/806r primers targeted for the V4 region of the 16S rRNA gene for bacteria and archaea and ITS-1/ITS-2 primers to amplify the ITS-1 spacer gene of 18S rRNA for fungi. Primers contained 12-bp barcodes unique to each sample and the appropriate adapters to permit sequencing on the Illumina Miseq platform. One-µl of genomic DNA was added to 13-µl of PCR-grade water, 10-µl of Prime Hot Master Mix, and 0.5-µl of reverse and forward primers and diluted with 1:10 PCR-grade water (Cutler et al., 2018). To denature DNA prior to amplification, reactions were held at for 3 minutes at 94 °C prior to subsequent amplification continuing for 35 cycles for 45 seconds, 60 seconds, and 90 seconds, with an additional 10 minute extension at 94 °C, 50 °C, 72 °C, and 72 °C, respectively to confirm amplification. Negative controls were used to ensure no contamination of samples occurred. Each sample was amplified in triplicate, and amplicons were pooled, cleaned and normalized and composited in equimolar concentrations using ThermoFisher SequalPrep Normalization Plate kits (Grand Island, NY). Sequencing was conducted on

an Illumina MiSeq (2x150bp chemistry) at the University of Colorado's Next Generation Sequencing Facility. Sequences were clustered into operational taxonomic units (OTUs) at the >97% sequence similarity level with the taxonomic identity of each OTU determined using the RDP classifier with a threshold of 0.5 (Wang et al., 2007) trained against either the Greengenes database for bacterial and archaeal 16S rRNA gene sequences (McDonald et al. 2011) or the UNITE database for fungal ITS sequences (Kõljalg et al. 2013).

Statistical Analyses for Effects of Environmental Factors (Soil Temperature and Moisture) and Treatments on r*E. coli* Populations

A one-way repeated measures analysis of covariance and Bonferonni post hoc tests were performed to compare effects of compost treatment on populations of r*E. coli* with temperature and moisture as co-variables. These statistical analyses were also applied to determine contamination of r*E. coli* onto edible produce. Linear mixed models used bivariate Pearson correlation tests to determine statistically significant relationships between r*E. coli* population, soil temperature and soil moisture (kPa). Chi-square analysis was used to determine significance at bag enrichment. IBM SPSS Statistics 24 software was used for the analysis of covariance, correlation, and chi-square analyses. Test for normality was computed prior to running statistical analyses.

Statistical Analyses for Effects of Microbial Populations (OTUs), Environmental Factors, and Treatments on r*E. coli* Through Time

Pairwise dissimilarity indices were computed using Bray-Curtis. Biplots were illustrated using principal coordinates analysis (PCO) and analyzed by permutational

multivariate analysis of variance (PERMANOVA) for statistical significance. PCO were completed to identify patterns of microbial community similarity among treatment and between fields. Permanova is a multivariate analysis using a covariate matrix by converting potentially linear correlated variables into uncorrelated variables using orthogonal transformation. All tests were performed using Primer 7 software (city, state, country).

Multivariate redundancy analyses (RDA) were used to summarize the impact of environmental factors on microbial soil communities using a repeated measurement design (Van den Brink et al., 2003). Principal response curves (PRC) analyzed the impact of compost on microbial communities through time. PRC displays changes in OTU values over time and each factor is represented as its own response curve in the plot relative to the control. Canoco 5 software was used for RDA and PRC analyses. (Van den Brink et al., 2003). Sequence data were rarified by randomly subsampling 4,000 and 10,000 reads per sample of respective 16S and fungal ITS datasets prior to computing downstream analyses.

Statistical Analyses for Effects of Ecoenzymatic activity (EEA) and Nutrients on r*E. coli* Populations

A linear mixed model ANOVA and Bonferonni post hoc tests was used to analyze the effect of compost treatment on nutrient availability to microbial communities. The ratio of BG to AP (C:P) is plotted on the *x*-axis and BG to the sum of NAG and LAP (C:N) ratio on the y-axis. Carbon availability was estimated as a vector length, calculating the square root of the sum of squared values of *x* and *y*, where *x* is C:P and *y* is C:N; Length= SQRT

($x^2 + y^2$) (Moorhead et al. 2013). Values > 1.0 suggest a carbon limitation. If carbon is unlimited (values < 1.0), then nitrogen or phosphorus is limiting as determined by computing the angle as the arc-tangent of the line between the plot origin and the data point; Angle (degrees)= DEGREES (ATAN2(x,y)) (Moorhead et al., 2013). Excel software was used to compute the vector length and angle and GraphPad Prism software to illustrate vector length and angle through time by treatment within each year. Complete linear mixed models were used to analyze the statistical significance of main effects between treatments as independent variables and covariables (dependent variables) such as environmental factors, C:N, C:P, Angle, and Vector Length.

Results

Effects of Compost on r*E. coli* Survival in Soils

Regardless of soil composition or treatment, *rE. coli* populations declined over time to 104 dpi. Generally, populations of r*E. coli* remained at higher levels in poultry pellet (PP) and poultry litter (PL) amendments when compared to DC and NC plots (Figure 1 and 2). Initial populations ranged from 2.5 to 4.5 $\log_{10}$ CFU/gdw when inoculated on Day 0. Levels of *rE. coli* increased slightly at 3 dpi for both fields and then declined exponentially for Year 1 and Year 2 at 104 and 102 dpi, respectively. For Year 1, PL, DC and NC treatments demonstrated declines of 1.07-1.21 log CFU/gdw, 3.42-4.29 log CFU/gdw, and 3.05-3.29 log CFU/gdw, respectively. Year 2 showed similar trends, where PP, PL, DC, and NC treatments demonstrated declines of 1.24-1.69 log CFU/gdw, 2.52-4.36 log CFU/gdw, 3.82-3.85 log CFU/gdw, and 4.74-4.88 log CFU/gdw, respectively. During Year 1, at 104 dpi, inoculated r*E. coli* survived at higher

populations in PL plots (-0.04 to 2.07 log MPN/gdw) in comparison to DC plots (-0.06 to -0.88 log MPN/gdw) and NC plots (-0.56 to -0.89 log MPN/gdw) (Figure 1). Similar trends were observed for Year 2, where at 102 dpi inoculated r*E. coli* survived at higher populations in PP plots (2.44 to 2.84 log MPN/gdw) and PL plots (below detectable levels -2.52 log MPN/gdw) in comparison to DC plots (-0.52 to 0.87 log MPN/gdw) and NC plots (-0.85 log MPN/gdw) (Figure 2). It is important to note that r*E. coli* levels in PL compost amended soils in Field A did go below detectable enumeration levels, while *E. coli* populations in Field B field did not. However, even though PL and PP treatments allowed greater levels of *rE coli* survival, *rE. coli* enumeration results for all treatments were statistically significant for both years combined (Table 3).

38 (of 526 total) soil samples that achieved bag enrichment and persisted beyond 102 (Year 1) or 104 (Year 2) dpi, the majority were NC and DC treatments, where of the 19 and 14 total samples that achieved bag enrichment, 17 (89.5%) and 12 (85.7%) samples were positive, respectively (Table 4). In contrast, PL and was PP did not commonly achieve bag enrichment, where of the 4 and 1 total samples that achieved bag enrichment, no samples (0%) and 1 (100%) sample tested positive for r*E. coli*, respectively.

Effects of Environmental Factors on Enumeration and Bag Enrichment r*E. coli* Results in Soils

Populations of r*E. coli* were independent of soil type, where all environmental factors (temperature at 10cm, water potential at 10 cm depth, and percent moisture did not have a statistically significant influence on r*E. coli* levels (CFU and MPN/g) in compost-amended soils and were relatively stable with some noted fluctuations ($p>0.05$) (Table 5).

However, percent moisture as a co-variate demonstrated that PP and P treatments were significantly different from DC and NC treatments, but those treatment pairs had no significant differences. Moisture content at 10 cm and temperature at 10 cm as co-variates both demonstrated that PP and P were significantly different from all other treatments, while DC and NC did not have significant differences.

Listeria spp. Recovery from Soil and Crop Samples

All compost soil treatment samples tested negative for *Listeria* spp., with the exception of a *L. innocua* isolate being detected in a loamy field control (without compost and without r*E. coli*, control plot). No radish samples tested positive for the presence of *Listeria* spp. after enrichment.

Effects of Composts and Environmental Factors on *E. coli* Contamination of Edible Crop

Enumeration results of radish samples demonstrated that the PL treatment (0.342-2.79 log MPN/radish sample) promoted the greatest level of r *E.coli* survival when compared to DC (undetectable to 1.41 log MPN/radish sample) and NC (undetectable to 0.785 log MPN/radish sample) treatments (Figure 3). While NC and DC treatments saw a gradual decline in r*E. coli* contamination of radish samples over time, PL actually showed a significant increase over time up to day 53 post inoculation, even though r*E. coli* levels decreased in soils ($p<0.05$) (Table 3).

Similar findings were shown for radish samples that achieved bag enrichment and persisted to the final harvest on 102 (Year 1) or 104 (Year 2) dpi (Table 4). Of the 27

NC radish samples that achieved bag enrichment, 21 (77.8%) of samples were positive for r*E. coli*, However, of the 6 samples that achieved bag enrichment for both DC and PL treatments, only 2 (33.3%) and 4 (66.7%) samples were positive for r*E. coli.*

Radish results demonstrated that temperature at 10 cm, water potential at 10 cm, and percent moisture were all statistically significant co-variates, where PL was significantly different from NC and DC (Table 6). When water potential and percent moisture are analyzed as covariates, DC and NC *E. coli* levels increased over time, while PL decreased. *E. coli* levels with temperature as co-variate observed the opposite effect, where DC and NC decreased, and PL treatments increased.

Indigenous Microbial Community Activity, Microbial Consortia and Ecoenzyme Function

There were main effects of compost on microbial ecoenzyme function but not field and year (Figure 4). Microbial communities were universally carbon limited across treatments in both fields for both years (vector length > 1, Figure 5). Vector length related to carbon deficiency was significantly different by year, but not by field or treatment (Figure 5), where a greater number of samples were carbon sufficient during Year 2 since their vector lengths did not exceed 1. Angles were statistically significant for all main effects ($p<0.05$) and interactions between compost by year ($p<0.05$), where main differences between PL and NC during Year 1, and PL to NC and DC during Year 2, were statistically significant (Figure 4). The majority of samples from both years exceeded a 45° angle, demonstrating P deficiency. In Year 1, samples tended to be N deficient initially and then shifted to more P deficient after 14 dpi (Figure 4). Results for Year 2 showed that samples were consistently P limited throughout the trial and also C

limited. This change after treatment was applied demonstrates that compost provides sufficient nutrients for *E. coli* growth but was limited in carbon throughout the experiment.

Effect of Indigenous Microbial Community on *E. coli* Survival

Bacterial and fungal communities were distinct for each field and were clustered by treatment during both years through the duration of both experiments. Composition of bacterial communities in PL treatments often contrasted to those of DC and NC treatments throughout all years ($p<0.05$) (Figure 6), with the exception of the composition of fungal communities in DC treatments contrasting with NC treatments in Field B plots. There was overlap in community composition of both bacterial and fungal communities in DC and NC treatments in both fields for all three years. For both years, bacteria and fungi composition was significantly different by treatment within field within year ($p<0.001$).

Members of *Bacteriodetetes* were found in all treatments, while *Acidobacteria*, *Proteobacteria*, and *Verrucomicrobia*, were more abundant in DC and NC in both fields. Members of *Chloroflexi*, TM6, and *Crenarchaeota* were found only in Lilac plots and were heavily populated in DC treatments (Figure 7). In contrast, *Gemmatimonadetes* was only found in Field B plots (RDA) and were most abundant in NC and DC plots (Figure 7). *Bacteriodetes* and *Proteobacteria* were abundant throughout both trials in both fields.

Fungal community composition demonstrated that all OTUs were relatively more abundant in DC and NC plots when compared to PL plots. While members of

Ascomycota and Fungi (unclassified) were found in both fields (Figure 7), members of *Zygomycota* were found only in Field A plots and *Basidiomycota* and unidentified members were only found in Field B plots (Figure 7).

Discussion

Compost is promoted as a safer and more sustainable approach in comparison to raw manure (U.S. FDA, 2018b). The FDA has even considered eliminating the 45 day application interval for composts that are treated properly, with the understanding that compost is a BSAAO that poses a reduced public health risk (U.S. FDA, 2014). The FDA also encourages application of FSMA-compliant compost to soils for production of fresh produce over manure due to reduced risk of pathogen contamination on the harvested produce when other stipulations in the Produce Safety rule are also implemented.

Poultry litter or dairy manure are used as agricultural fertilizers to provide nutrients to crops. In 2005, 132 million metrics tons of dairy manure was applied to roughly 9.2 hectares of farmland (Edrington et al., 2009). Results show that soils amended with dairy manure would meet the microbiological standards established by the U.S EPA under Part 503 of the biosolids rule by 28 days, while poultry litter and poultry pellet amended soils would have to be held for greater than 56 days to achieve this same standard. Prior to application, composts tested negative for resident *E. coli* and *Listeria* spp., which is expected for treatments that go through thermophilic heat treatment. This study only found indigenous *Listeria* spp. in control plots, suggesting that *Listeria* spp. are present in soils regardless of compost amendment application.

Several studies have found that temperature, high humidity, and pathogen concentration can affect produce contamination (Park et al., 2012). Our results are consistent with other research demonstrating that *E. coli* O157:H7 can persist in manure amended soils from 154-217 days when inoculated with large concentrations (10^7 CFU/g) (Patel et al., 2009) and can contaminate edible crops for up to 77 days and 168 days respectively (Patel et al, 2009; Cooley, 2007). Our results agree with reports on lettuce on which attenuated *E. coli* O157:H7 could be detected on lettuce plants grown in inoculated soil amended with manure composts for up to 77 days and in soils for up to 126 days (Islam et al., 2004). Another study detected *E. coli* O157:H7 on lettuce 7 days after inoculation when the inoculum was applied higher than 6 log CFU/ml (Erickson et al, 2010). *E. coli* O157:H7 persisted on the abaxial surface (underside) of the leaves for longer than the adaxial surface. Although findings are consistent with Cutler et al. (2018) and (Sharma et al. 2016), who observed longer periods *E. coli* persistence in soils amended with PL compost, it is unclear as to why PL promoted *E. coli* growth between 56 dpi and 102 dpi on radish crops. Some studies suggest that *E. coli* survivorship on pre-harvest produce varies depending on environmental conditions (Markland et al. 2012, Weller et al. 2017).

Our results validate other reports suggesting that *E. coli* is an inappropriate indicator species for *Listeria* spp. presence in soils (Lekkas et al., 2016; Brochier et al. 2012). Survival of *Enterococcus* spp. in soils was similar in soils either unamended or amended with compost (Brochier et al., 2012). In the same study, *L. monocytogenes* was nondetectable by direct plating due to lack of presence or levels being below detectable limits (Brochier et al., 2012). Overall, quantitative risk assessments of microbial die off

rates can be used to better identify intervention and preventive strategies to control food safety risks associated with raw produce (Wood et al., 2010). Survival rates are ideal risk models to estimate potential contamination levels of produce at time of harvest. Survival rates of pathogens in challenge studies on produce and reported die off rates of *E. coli* with ranges of 0.4 to 1.64 log CFU/day. One study observed 0.54 to 1.64 log CFU/day on spinach greens in Nova Scotia, Canada (Wood et al., 2010). Soils amended with bovine manure decreased survival rates of *S. enterica* serovar Typhirium, on leafy greens like spinach (Natvig et al., 2002).

Survival rates can be influenced by the physical environment of certain produce surfaces, which can be considered inhospitable for the growth and survival of bacteria, depending upon the lack of nutrients, availability of free moisture, temperature and humidity fluctuations, and ultraviolet light (Harris et al., 2003). Certain conditions, such as sunlight for example, can damage and lyse the bacterial cells due to ultraviolet light. However, free moisture on leaves from various precipitations, such as rainfall, dew, or irrigation, may promote persistence and growth of microbial populations (Park et al., 2012). Many studies report that warm temperature, high humidity, and pathogen concentration can affect produce contamination (Park et al., 2012). For example, pathogen survival declines in manure (compared to BSAAO) when temperatures increased between 7°C to 33°C (Park et al., 2012; Sharma and Reynnells, 2016; Semenov et al., 2007; Van Elsas et al., 2011). Warm temperatures also favored growth or maintained pathogens on produce as a result of biofilms that offer protection and buffer them from environmental extremes (Park et al., 2012). *E. coli* strains attributed to outbreaks on produce, such as leafy greens, are given the opportunity to adapt to such

stressors in the environment pre-harvest, leading to persistence (Markland et al., 2013). Therefore, once in food or food ingredients, *E. coli* O157:H7 has the ability to survive when stored under refrigeration temperatures and has a high acid tolerance (Islam et al., 2004). Biofilms are also able to be formed on fresh produce as bacterial cells may aggregate and protect cells from environmental stressors (Olaimat and Holley, 2012). Contamination trends are of pathogens onto produce is crucial as damage on handled or fresh cut produce enables pathogen persistence, especially at non-refrigerated temperatures (Park et a. 2012; Harris et al., 2003). *L. monocytogenes* can grow on vegetables under refrigerated and ambient temperatures and on non-acidic fruits (Harris et al., 2003), however *L. monocytogenes* has also been detected on the surface of tomatoes (Beuchat and Brackett, 1991).

Produce conditions including plant age, leaf age, physical damage and epiphytic bacteria are also correlated with produce contamination (Park et al., 2012). Mature produce intended for harvest are more susceptible to contamination by *Salmonella* spp. and *E. coli* O157:H7 due to longer bouts of exposure time. In contrast to findings determined by Moyne et al. (2013), studies have found that sprinkler irrigation often led to more bacterial growth on lettuce than drip irrigation, which was attributed to greater availability of free water (Williams et al., 2013).

Soil moisture correlates positively with survival of *E. coli* in manure-amended soils (Lekkas et al., 2016). Soil moisture and temperature tend to impact the availability of N, especially in sandy soils (Cutler et al. 2018, Jamieson et al. 2002), where NH_4-N is converted to NO_3-N through nitrification. Dairy manure provides high nitrogen as NO_3-N (Islam et al. 2004, Cutler et al., 2018, Jack 2011). However, *E. coli* prefers the

NH_4-N form (Reitzer 2003), especially when bioavailability is high (Franz et al. 2008). N in the form of NO_3-N is more likely to leach but NH_4-N can bind to clay and other organic materials and is thus less prone to leaching (Paul 2015). This at least partly explains why total N availability is available for longer periods of time in poultry litter composts when compared to dairy composts (Cutler et al., 2018). Poultry litter also has higher protein and amino acid content resulting in greater N availability as NH_4. Sharma et al (2016) had also found similar results, reporting higher nutrient content (nitrogen: phosphorus) in poultry litter amendments that are likely attributing to the extended survival of generic *E. coli* and O157:H7.

As previously mentioned, moisture is considered a factor that determines the survival of *E. coli* in clay soils (Fremaux et al., 2008; Jamieson et al., 2002). The association between organic matter particle size and distribution affects die off rates of *E. coli* as water potential exceeds equilibrium in a system when other extrinsic factors (i.e. temperature) are the same (Whalley et al., 2013). Survival curves of *E. coli* persisted for longer in clay soils than in sandy soils, consistent with findings reported by Lekkas et al., (2016). The variation in slope between sites (Field A had a 3-8% slope while Field B had a slope of 5-12%) and shade may have had an impact on *E. coli* survival due to impacts on moisture retention, causing a slower *E. coli* mortality (Zaleski et al., 2005). Also as previously mentioned, free moisture on leaves from various precipitations, such as rainfall, dew, or irrigation (Park et al., 2012), soil type and location, may promote persistence of enteric microbial populations (Lau and Ingham, 2001; Jamieson et al., 2002; Zalenski et al., 2005).

Moisture also promotes *E. coli* survival through the creation of an anaerobic environment that allow facultative anaerobic *E. coli* to undergo metabolic respiration (Tiedje, 1984). Therefore, *E. coli* bacteria are able to take advantage of the competitive circumstances and thrive in the absence of indigenous obligate aerobes (Cutler et al., 2018). As moisture declines and temperatures increase during the summer months, *E. coli* cannot survive and populations fall below detectable levels. Less negative water potential (wetter soils) can also lead to the release and diffusion of nutrients, which very likely impacts the microbial community by enabling a change from aerobes to anaerobes and subsequently to facultative aerobes (Cutler et al., 2018).

Although confounded with slope and landscape, soil type did not significantly alter the survival of *E. coli i*n our experiment. Soil type has been reported to change survival of *E. coli* O157:H7 (Jamieson et al., 2002; Reynells et al., 2014) or have no effect (Sharma et al., 2016) . Loamy soil contains greater organic matter content and clay, both which hold moisture and organic carbon and drain more slowly than sands, favoring survival and re-growth of *E. coli.* Organic amendments to soil, as manure or composted manure, changes the form and availability of N and P. In fresh poultry manure, 60-80% of N is in organic form as proteins and amino acids (DeLaune et al. 2004). Organic nitrogen degrades releasing NH_4-N. This can at least partly explain why both levels of total N and *E. coli* survival endured longer in poultry than other compost treatments.

Long term *L. monocytogenes* survival also depends heavily on soil texture and clay content survived up to 84 days in 71% of soils tested (Locatelli et al., 2013). *L. monocytogenes* will persist for longer durations of time in a fertile soil when compared to a clay soil. This has led to contamination of *L. monocytogenes* from amended soils onto

produce and seeds of crops such as carrots, lettuce, radish, spinach, and tomato (Locatelli et al., 2013), due to its presence in vegetation, water, sediment and soils. However, the pathogen was more abundant in clay soils when compared to sandy soils (Locatelli et al., 2013).

Conclusions

The soil type, geographical location, and biotic and abiotic factors all impact the survival of *E. coli* populations in compost amended soils. While poultry litter is ideal for crop utilization because it provides high levels of N primarily as NH4 in a mineralized form, this study is consistent with other studies conducted in other regions of the US that show that poultry litter-based BSAAO support greater numbers and longer periods of persistence in field soils of *E. coli* than dairy-based BSAAO ( )Shiga-toxin producing *E. coli* has been a cause of many outbreaks related to produce and this research provides findings that will help regulators and farmers consider alternative practices when harvesting produce intended for consumption.

Table 1: Soil characteristics for experimental sites: Field A and B (Lekkas et al., 2016)

Field	***Soil Type***	***Slope***
A	*Hinesburg B Sandy Loam (Sand: 60%, Silt:10%, Clay: 30%)*	*3-8%*
B	*Adams B Loamy Sand (Sand: 40%, Silt: 40%, Clay: 20%)*	*5-12%*

Table 2: Enzymes tested and associated soil substrates, experimental substrates, and positive controls (Culter et al., 2018)

Enzyme	Organic Substrate (Target Nutrient)	Substrate Used	Positive Control
β-1,4-glucosidase (BG)	Cellulose (Carbon)	4-MUB- β-D-glucoside (Sigma #M2133)	4-methylumbilliferyl (Sigma #M1381)
Phosphatase (AP)	Phosphomonoesters (Phosphorous)	4-MUB-phosphate (Sigma #M8883)	4-methylumbilliferyl (Sigma #M1381)
β-1,4-N-acetylglucosaminidase (NAG)	Chitin (Carbon and Nitrogen)	4-MUB-N-acetyl- β-glucosaminide (Sigma #2133)	4-methylumbilliferyl (Sigma #M1381)
Leucine (LUC)	L-leucine aminopeptidase (Nitrogen)	L-leucine-7-amido-4-methylcoumarin (Sigma #L2145)	7-amido-4-methylcoumarin (Sigma #A9891)

Table 3: Linear equations of rifampicin resistant *E. coli* (*rE. coli*) enumeration results from soil and radish samples processed during Year 1 and Year 2 summer trials

Habitat			Linear Regression Models	
	Treatment	Y=	Slope (a)	Intercept (b)
Soil: Log CFU and MPN/gdw*	NC[a]	Y=	-0.037± .003*	3.101± .101*
	DC[b]	Y=	-0.032± .003*	3.330± .099*
	PL[c]	Y=	-0.027± .002*	4.208± .098*
	PP[d]	Y=	-0.021± .004*	4.736± .147*
Radishes: Log MPN/sample*	NC[a]	Y=	-0.008± .027	0.105± 1.103
	DC[a]	Y=	-0.027± .016	1.421± .682*
	PL[b]	Y=	0.051± .016*	-0.732± .682

Linear models: one-way ANOVA, Bonferroni alpha (*$p < 0.05$).
Subscripts explain statistically significant differences between treatments.
NC= no compost, DC= dairy compost, PL= poultry litter compost, PP=poultry pellets.
CFU/gdw= colony forming unit per gram of dry weight.
MPN/gdw= most probable number per gram of dry weight.

Table 4: Statistical significance of rifampicin resistant *E. coli* (*rE. coli*) bag enrichment (BE) results from soil and radish samples processed during Year 1 and Year 2 summer trials

Habitat	Treatment[a]	Presence (+)/ Total Samples (%)
Soil: BE/gdw*	NC	17/19 (89.5)
	DC	12/14 (85.7)
	PL	0/4 (0)
	PP	1/1 (100)
Radishes: BE	NC	21/27 (77.8)
	DC	2/6 (33.3)
	PL	4/6 (66.7)

[a]Chi-square analysis was used to determine statistical significance.
*BE data were found to be statistically significant; p=0.001.

Table 5: Linear equations of rifampicin resistant *E. coli* (*rE. coli*) enumeration results (log CFU and MPN/gdw) from soil samples processed and independent variable interactions during Year 1 and Year 2 summer trials

Independent Variables			Linear Regression Models	
	Treatment	Y=	Slope (a)	Intercept (b)
Moisture Content (% water content)	NC[a]	Y=	0.132± .033*	0.695± .428
	DC[a]	Y=	0.148± .034*	0.696± .450
	PL[b]	Y=	0.110± .020*	2.099± .270*
	PP[b]	Y=	-0.003± .083	4.165± 1.270*
Water potential at 10cm depth (kPa)	NC[a]	Y=	0.007± .002*	2. 430± .120*
	DC[a]	Y=	0.011± .002*	2.697± .119*
	PL[b]	Y=	0.008± .002*	3.587± .116*
	PP[c]	Y=	-0.043± .041	4.044± .349*
Temperature at 10 cm depth (^{o}C)	NC[a]	Y=	-0.055± .034	3.409± .711*
	DC[a]	Y=	-0.74± .034*	4.001± .706*
	PL[b]	Y=	-0.097± .033*	5.411± .704*
	PP[c]	Y=	-0.098± .046*	6.025± .807*

Linear models and interactions: one-way ANOVA, Bonferroni alpha (*$p < 0.05$).
Subscripts explain statistically significant differences between treatments.
NC= no compost, DC= dairy compost, PL= poultry litter compost, PP=poultry pellets.
CFU/gdw= colony forming unit per gram of dry weight.
MPN/gdw= most probable number per gram of dry weight.

Table 6: Linear equations of rifampicin resistant *E. coli* (*rE. coli*) enumeration results (log MPN/gdw) and independent variable interactions from radish samples processed and independent variable interactions during Year 1 and Year 2 summer trials

Independent Variables			Linear Regression Models	
	Treatment	Y=	Slope (a)	Intercept (b)
Moisture Content (% water content)*	NC[a]	Y=	0.009± .029	-0.334± .399
	DC[a]	Y=	0.058± .075	-0.587± 1.094
	PL[b]	Y=	-0.248± .064*	5.205± .981*
Water potential at 10cm depth (kPa)*	NC[a]	Y=	0.002± .008	-0.172± .320
	DC[a]	Y=	0.014± .006*	0.620± .206*
	PL[b]	Y=	-0.014± .006*	1.086± .206*
Temperature at 10 cm depth (^{o}C)*	NC[a]	Y=	-0.033± .063	0.439± 1.284
	DC[a]	Y=	-1.181± .045*	4.056± .943*
	PL[b]	Y=	0.155± .045*	-1.809± .943

Linear models and interactions: one-way ANOVA, Bonferroni alpha (*p< 0.05).
Subscripts explain statistically significant differences between treatments.
NC= no compost, DC= dairy compost, PL= poultry litter compost, PP=poultry pellets.
MPN/gdw= most probable number per gram of dry weight.

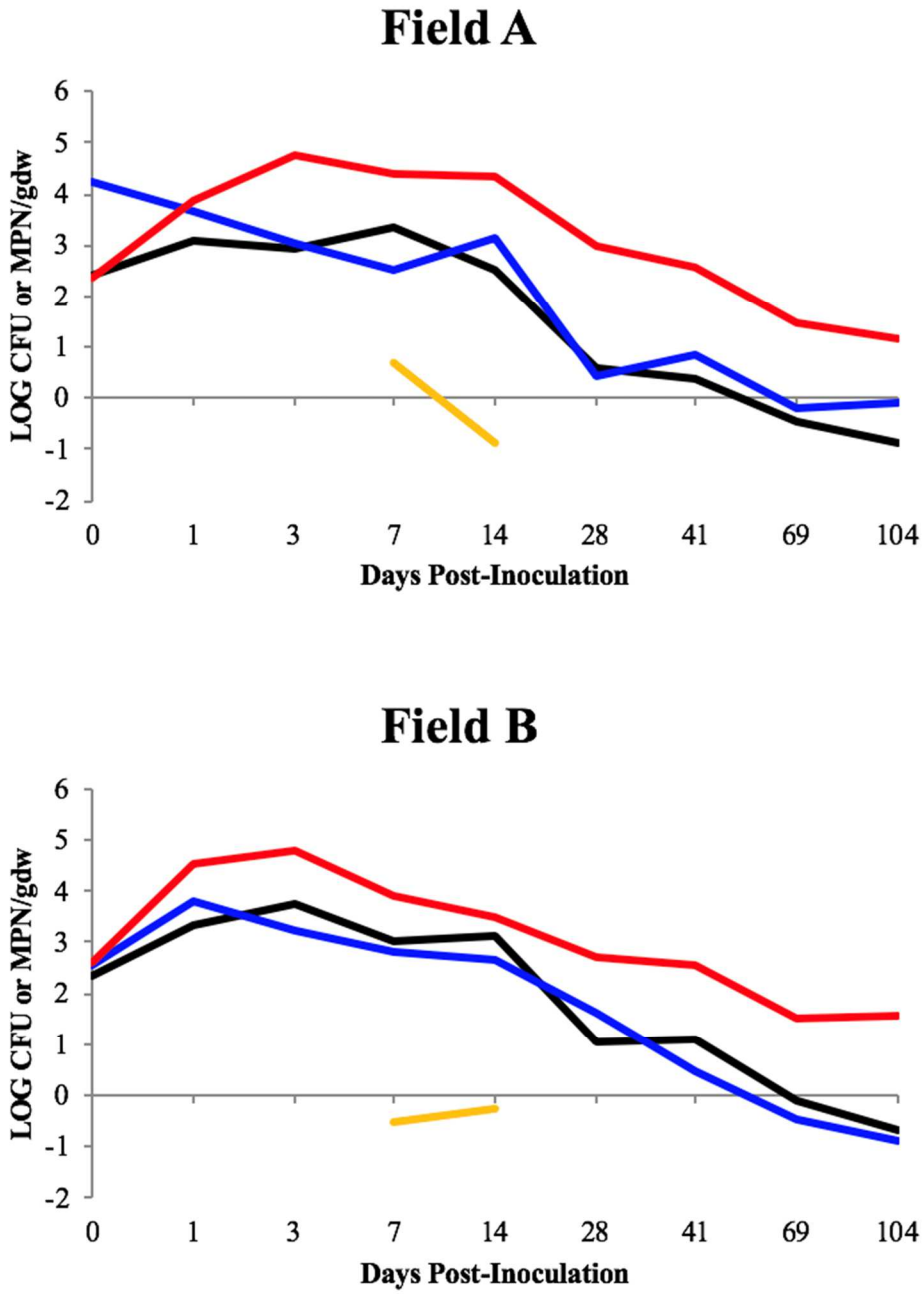


Figure 1: Year 1 *E. coli* populations through time in Field A (top) and Field B (bottom). Control= yellow, No Compost (NC)= black, Dairy Compost (DC)= blue, and Poultry Litter (PL)= red.

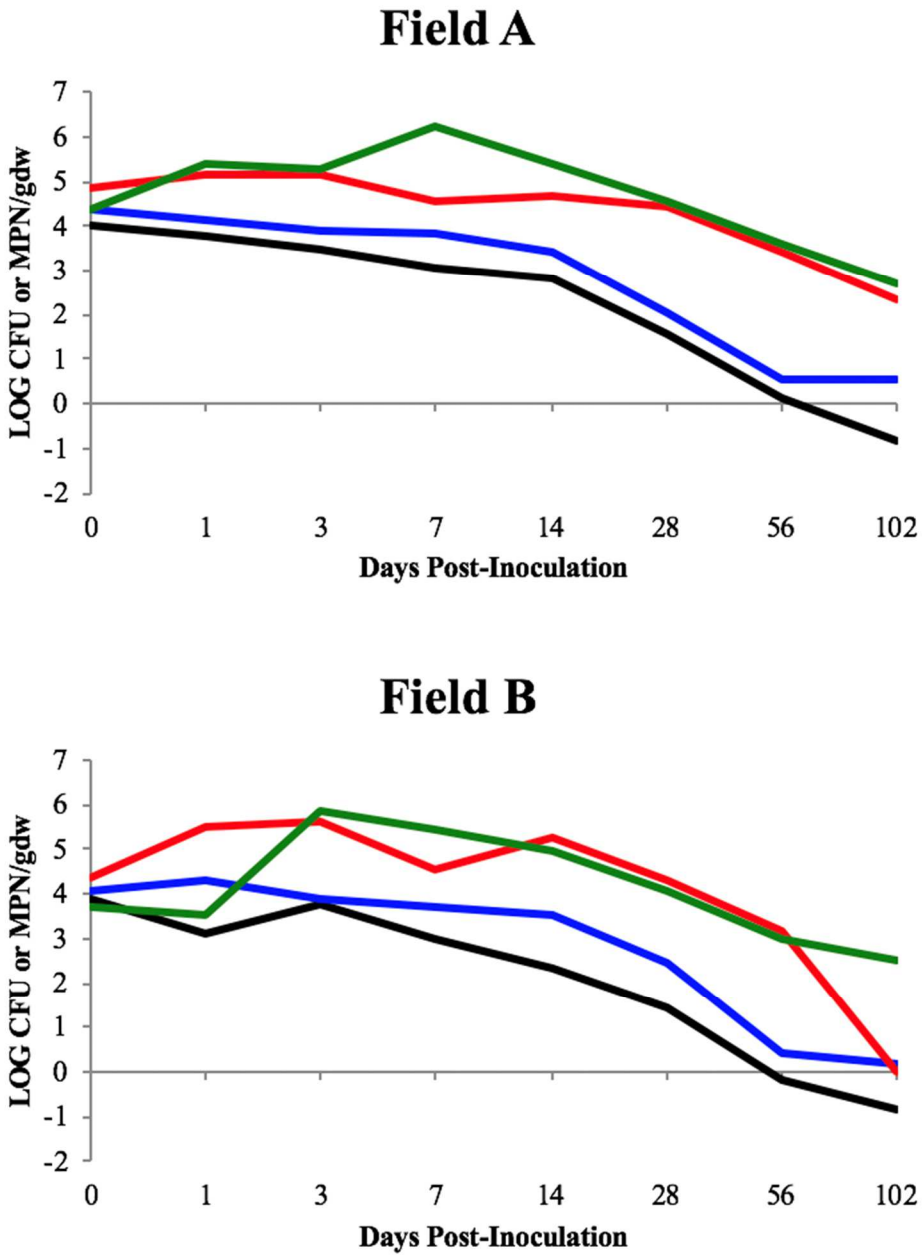


Figure 2: Year 2 *E. coli* populations through time in Field A (top) and Field B (bottom). No Compost (NC)= black, Dairy Compost (DC)= blue, and Poultry Litter (PL)= red, Poultry Pellets (PP)= green.

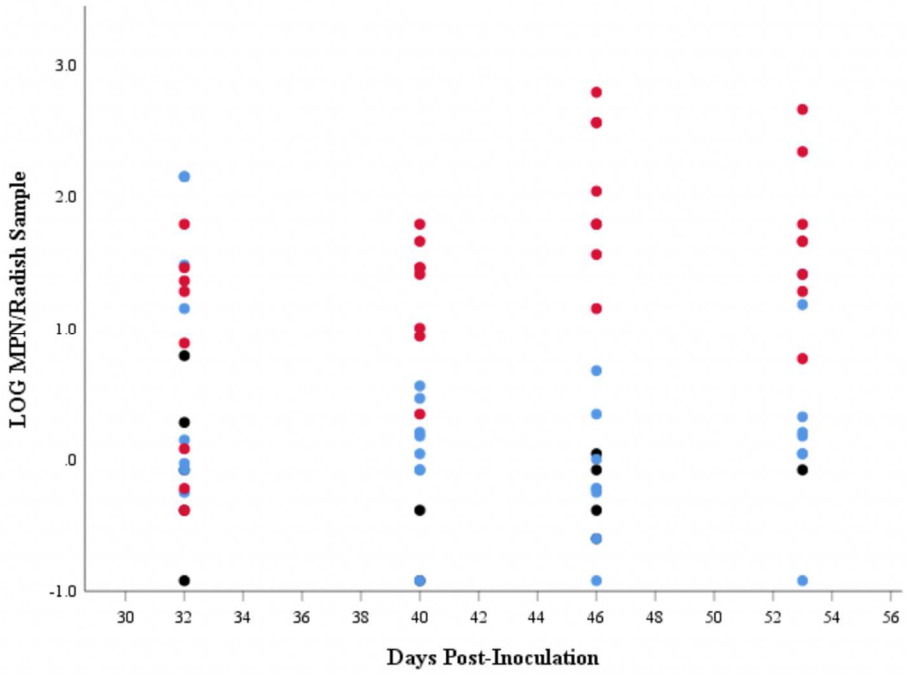


Figure 3: *E. coli* Enumeration Results of Radish Crop. Treatments: No compost (NC)= black, Dairy Compost (DC)= blue, and Poultry Litter Compost (PL)= red.

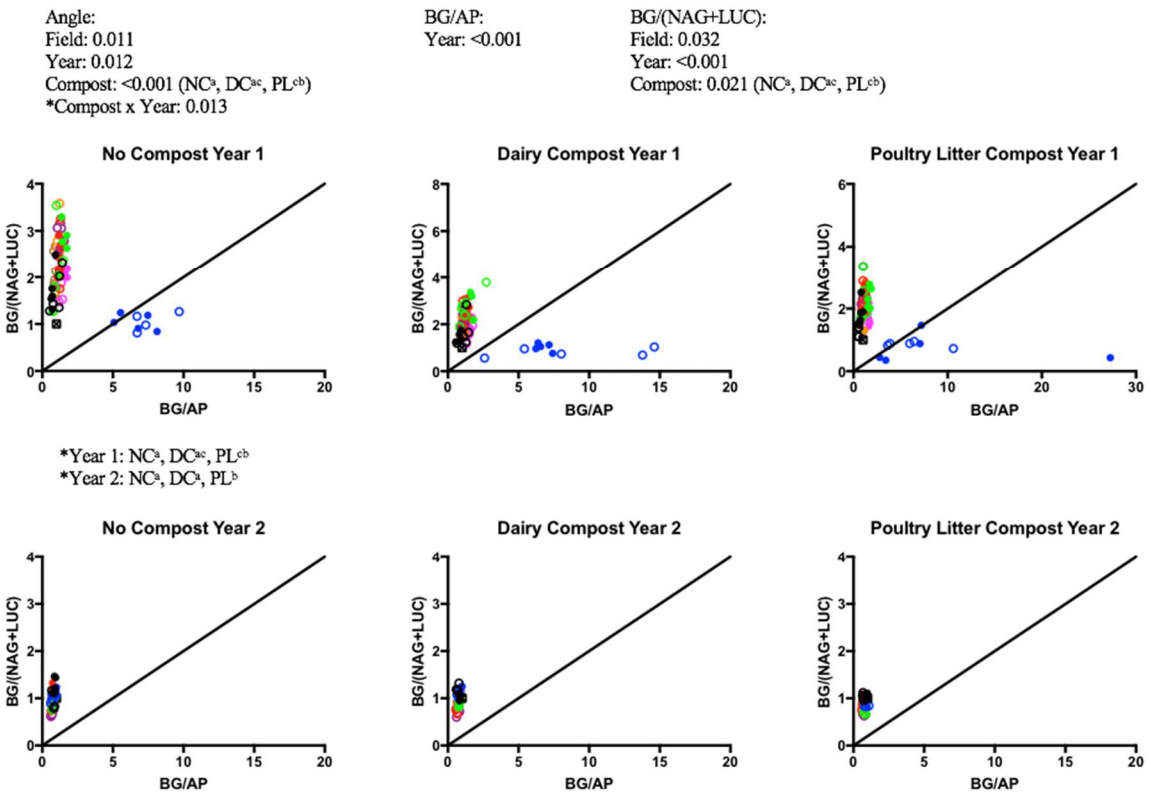


Figure 4: Ecoenzyme activity through time of BG/AP and BG/(NAG+LUC) by compost (No Compost (top), Dairy Compost (middle), Poultry Litter Compost (bottom) by year (Year 1 (left) and Year 2 (right). Fields are labeled as Field A soil (open circle) and Field B soil (closed circle). Days after inoculation are illustrated by contrasting colors: Black (3), Blue (7), Green (14), Red (28), Purple (DPI), Orange (DPI), Pink (77), Brown (84). Subscripts explain statistically significant differences between treatments. *=significance of treatments is associated with the compost x year interaction.

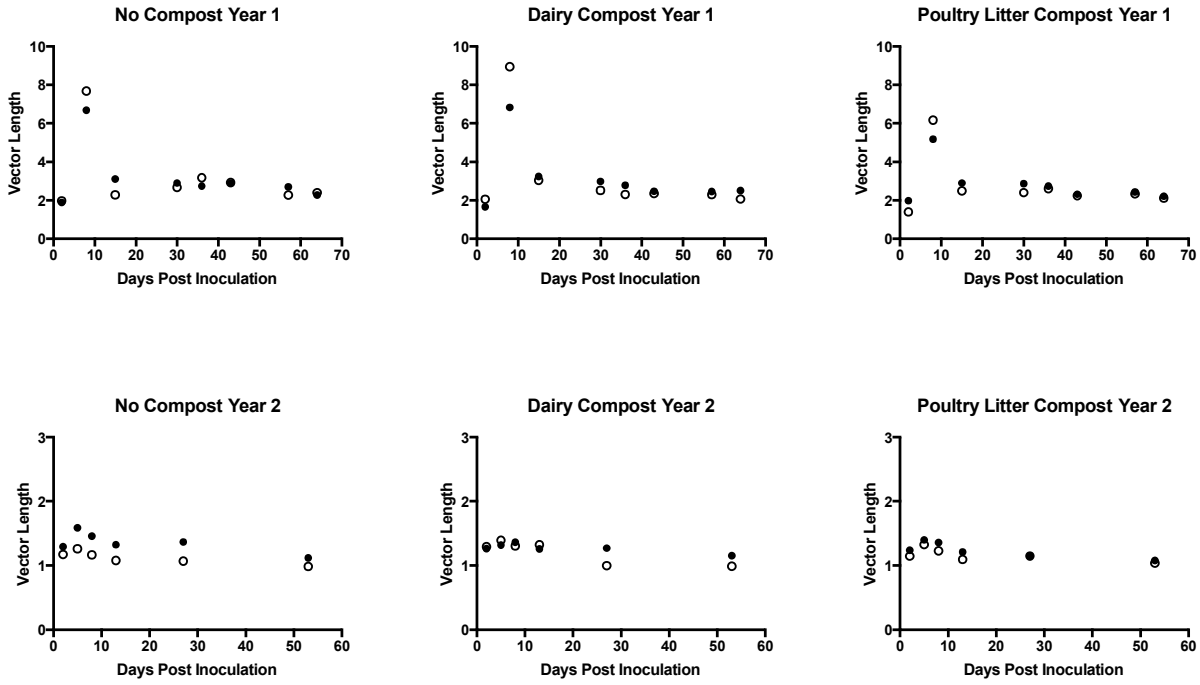


Figure 5: Vector length over time by compost (No Compost, Dairy Compost, Poultry Litter Compost) by year (Year 1 and Year 2). Year $p < 0.0001$. Fields are labeled as Field A soil (open circle) and Field B soil (closed circle).

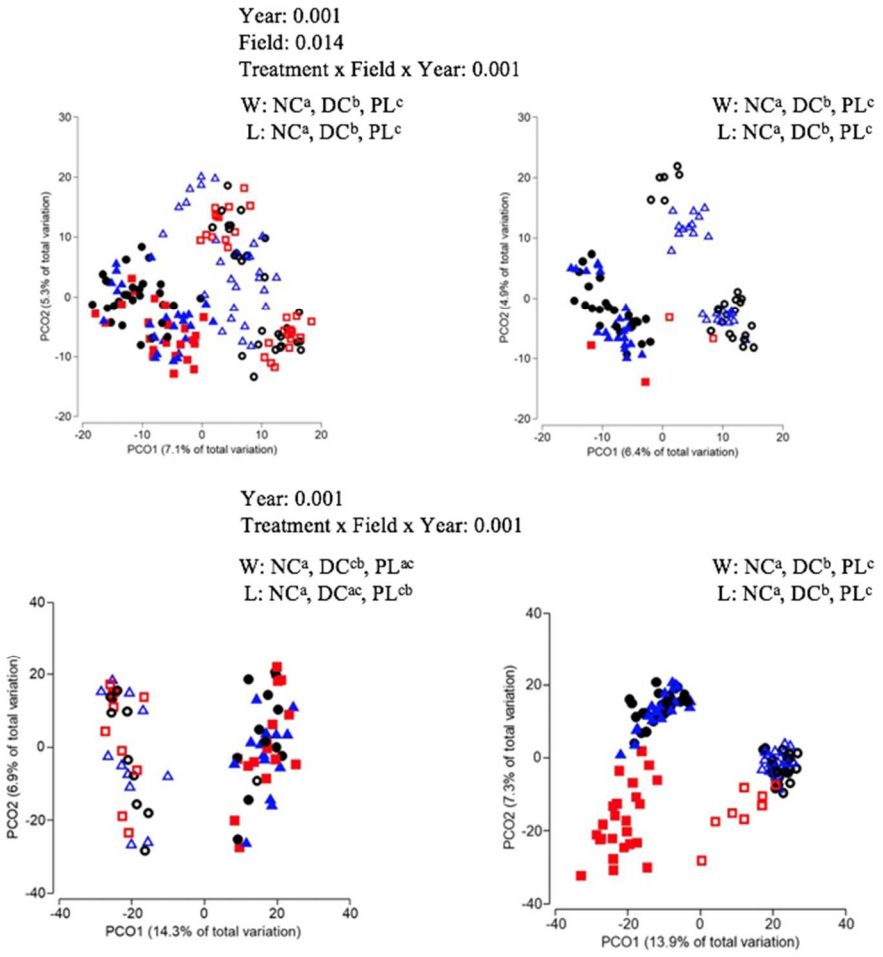
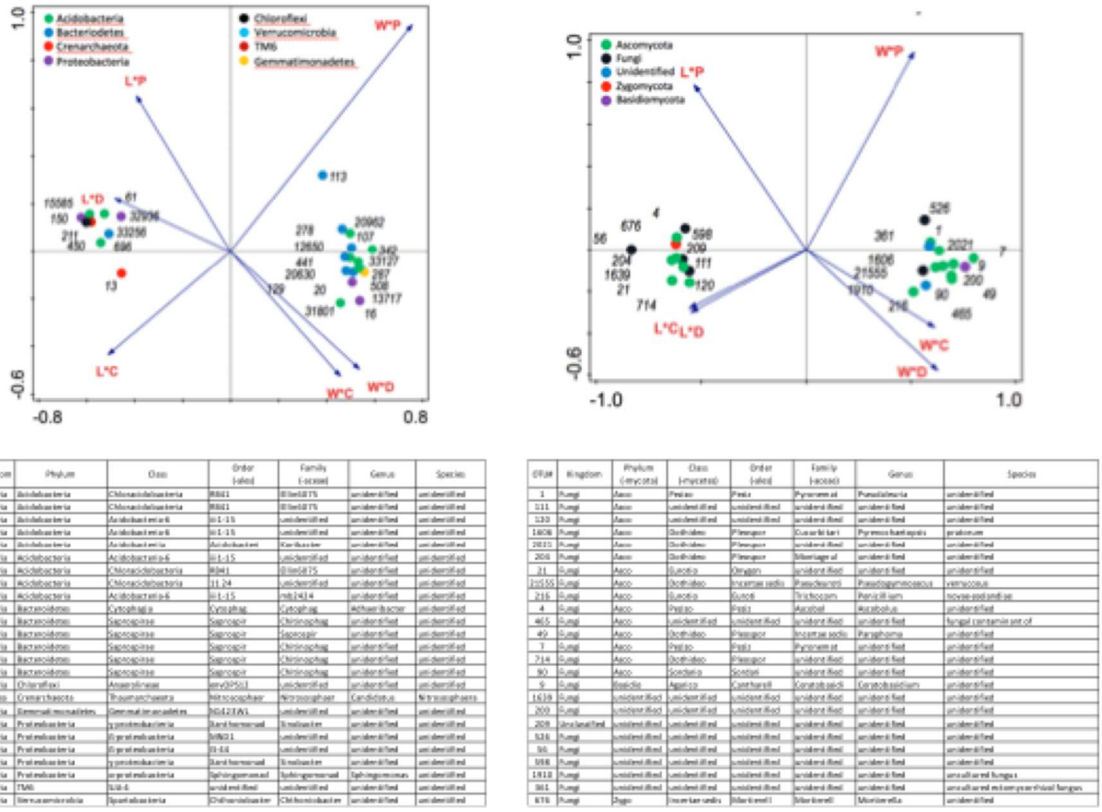


Figure 6: Principal coordinate analysis (PCO) based on Bray-Curtis dissimilarity of all 16S sequences for bacteria and archaea (top) and ITS sequences for fungi (bottom) for all compost treatments (poultry, dairy compost, none) combined (Year 1: left; Year 2: right). Symbols represent Bray-Curtis dissimilarity values between pairwise samples. Fields are labeled as Field A soil (open triangle) and Field B soil (closed triangle). Treatments are labeled as black is control, blue is dairy compost, red is poultry litter compost. Permutational analysis of variance P values are shown above each plot. Subscripts explain statistically significant differences between treatments.

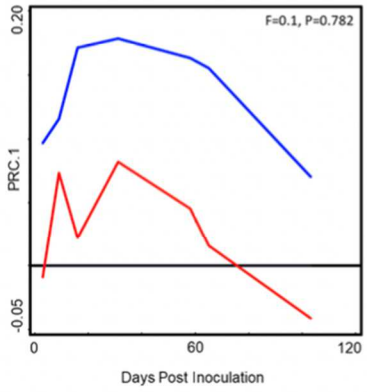


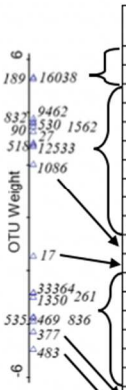
OTU#	Kingdom	Phylum	Class	Order (-ales)	Family (-aceae)	Genus	Species
187	Bacteria	Acidobacteria	Chloracidobacteria	RB41	Ellin6075	unidentified	unidentified
12893	Bacteria	Acidobacteria	Chloracidobacteria	RB41	Ellin6075	unidentified	unidentified
16717	Bacteria	Acidobacteria	Acidobacteria-6	iii1-15	unidentified	unidentified	unidentified
25585	Bacteria	Acidobacteria	Acidobacteria-6	iii1-15	unidentified	unidentified	unidentified
31801	Bacteria	Acidobacteria	Acidobacteria	Ko1-23	Koribacter	unidentified	unidentified
341	Bacteria	Acidobacteria	Acidobacteria-6	iii1-15	unidentified	unidentified	unidentified
441	Bacteria	Acidobacteria	Chloracidobacteria	RB41	Ellin6075	unidentified	unidentified
61	Bacteria	Acidobacteria	Chloracidobacteria	11-24	unidentified	unidentified	unidentified
686	Bacteria	Acidobacteria	Acidobacteria-6	iii1-15	ntl(2414	unidentified	unidentified
113	Bacteria	Bacteroidetes	Cytophagia	Cytophag	Cytophag	Adhaeribacter	unidentified
129	Bacteria	Bacteroidetes	Sapropspirae	Sapropspir	Chitinophag	unidentified	unidentified
20630	Bacteria	Bacteroidetes	Sapropspirae	Sapropspir	Sapropspir	unidentified	unidentified
20962	Bacteria	Bacteroidetes	Sapropspirae	Sapropspir	Chitinophag	unidentified	unidentified
278	Bacteria	Bacteroidetes	Sapropspirae	Sapropspir	Chitinophag	unidentified	unidentified
13256	Bacteria	Bacteroidetes	Sapropspirae	Sapropspir	Chitinophag	unidentified	unidentified
211	Bacteria	Chloroflexi	Anaerolineae	env20503	unidentified	unidentified	unidentified
13	Archaea	Crenarchaeota	Thaumarchaeota	Nitrosophaer	Nitrosophaer	Candidatus	Nitrosophaera
188	Bacteria	Gemmatimonadetes	Gemmatimonadetes	NO1231A1	unidentified	unidentified	unidentified
150	Bacteria	Proteobacteria	gammaproteobacteria	Xanthomonad	Xanthomon	unidentified	unidentified
16	Bacteria	Proteobacteria	deltaproteobacteria	bf801	unidentified	unidentified	unidentified
28	Bacteria	Proteobacteria	deltaproteobacteria	11-24	unidentified	unidentified	unidentified
12916	Bacteria	Proteobacteria	gammaproteobacteria	Xanthomonad	Xanthomon	unidentified	unidentified
31127	Bacteria	Proteobacteria	alphaproteobacteria	Sphingomonad	Sphingomonas	Sphingomonas	unidentified
150	Bacteria	TM6	SJA-6	unidentified	unidentified	unidentified	unidentified
287	Bacteria	Verrucomicrobia	Spartobacteria	Chthoniobacter	Chthoniobacter	unidentified	unidentified

OTU#	Kingdom	Phylum (-mycota)	Class (-mycetes)	Order (-ales)	Family (-aceae)	Genus	Species
1	Fungi	Asco	Pezizo	Peziz	Pyronemat	Pseudaleuria	unidentified
111	Fungi	Asco	unidentified	unidentified	unidentified	unidentified	unidentified
130	Fungi	Asco	unidentified	unidentified	unidentified	unidentified	unidentified
1808	Fungi	Asco	Dothideo	Pleospor	Cucurbitari	Pyrenochaetopsis	pratorum
2011	Fungi	Asco	Dothideo	Pleospor	unidentified	unidentified	unidentified
204	Fungi	Asco	Dothideo	Pleospor	Montagnul	unidentified	unidentified
21	Fungi	Asco	Eurotio	Onygen	unidentified	unidentified	unidentified
10155	Fungi	Asco	Dothideo	Incertae sedis	Pseudeurot	Pseudogymnoascus	verrucosus
216	Fungi	Asco	Eurotio	Eurot	Trichocom	Penicillium	novae-zeelandiae
4	Fungi	Asco	Peziz	Peziz	Ascobol	Ascobolus	unidentified
465	Fungi	Asco	unidentified	unidentified	unidentified	unidentified	fungal contaminant cf
49	Fungi	Asco	Dothideo	Pleospor	Incertae sedis	Paraphoma	unidentified
7	Fungi	Asco	Peziz	Peziz	Pyronemat	unidentified	unidentified
714	Fungi	Asco	Dothideo	Pleospor	unidentified	unidentified	unidentified
90	Fungi	Asco	Sordario	Sordari	unidentified	unidentified	unidentified
9	Fungi	Basidio	Agaricu	Cantharell	Craterocol	Craterocolla	cerasi
1618	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
200	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
209	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
526	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
56	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
598	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
1913	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
361	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
676	Fungi	Zygo	Incertae sedis	Mortierell	Mortierell	Mortierella	unidentified

Figure 7: Redundancy analysis (RDA) of A) 16S sequences (left) and B) ITS sequences (right) for Field A and B (Cutler et al. OTU data, Year 1 OTU data, and Year 2 OTU data, combined). This diagram summarizes the variation of OTU composition explained by factors, after covariate effects are taken into consideration. Distance between symbols approximates the average dissimilarity of OTU composition between the two sample classes based on the Euclidean distance. These sample classes are interpreted by individual symbols representing dummy variables that correspond to individual levels of a factor. Each arrow points in the direction where the largest increase of value for OTUs occurs. The angle between these arrows (alpha) indicate the sign of the correlation between OTUs, where a positive correlation is signified by a sharp angle, and a negative correlation is when the angle is larger than 90 degrees. The length of the arrow is a measure of fit for the OTU. The length of the arrows determines the multiple correlation of the OTU with the ordination axis. ITS: F=7.0, p=0.002. 16S: F=5.6, p=0.002. L=Field A, W=Field B, C= No Compost; D= Dairy Compost, P= Poultry Litter.

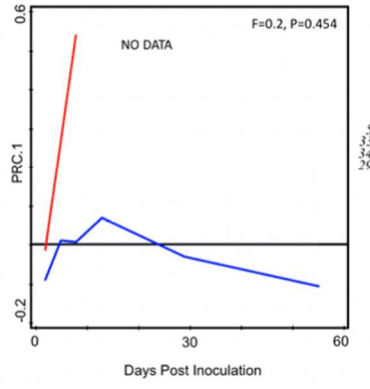
Appendix:

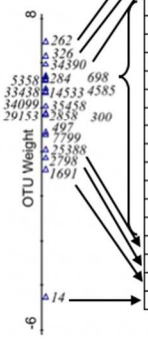




OTU#	Phylum	Class	Order (-ales)	Family (-aceae)	Genus	Species
189	Proteobacteria	ß-proteobacteria	Methylophil	Methylophil	Methylotenera	mobilis
16038	Acidobacteria	Chloracidobacteria	RB41	Ellin6075	unidentified	unidentified
832	Proteobacteria	Δ-proteobacteria	Myxococc	Haliangi	unidentified	unidentified
9462	Proteobacteria	Δ-proteobacteria	Myxococc	Haliangi	unidentified	unidentified
530	Bacteroidetes	Saprospirae	Saprospir	Chitinophag	Chitinophaga	unidentified
1562	Bacteroidetes	Cytophagia	Cytophag	Cytophag	unidentified	unidentified
90	Acidobacteria	Chloracidobacteria	RB41	unidentified	unidentified	unidentified
27	Acidobacteria	Chloracidobacteria	RB41	Ellin6075	unidentified	unidentified
518	Chloroflexi	Anaerolineae	SBR1031	A4b	unidentified	unidentified
12533	Acidobacteria	Acidobacteria-6	iii1-15	unidentified	unidentified	unidentified
1086	Acidobacteria	BPC102	unidentified	unidentified	unidentified	unidentified
17	Acidobacteria	Acidobacteria-6	iii1-15	unidentified	unidentified	unidentified
33364	Acidobacteria	Acidobacteria-6	iii1-15	unidentified	unidentified	unidentified
261	Acidobacteria	Acidobacteria-6	iii1-15	unidentified	unidentified	unidentified
1350	Acidobacteria	Acidobacteria-6	CCU21	unidentified	unidentified	unidentified
535	Actinobacteria	Thermoleophilia	Solirubrobacter	unidentified	unidentified	unidentified
469	Proteobacteria	α-proteobacteria	Rhizobi	Hyphomicrobi	Pedomicrobium	unidentified
836	Proteobacteria	α-proteobacteria	Rhizobi	Rhodobi	Afifella	unidentified
377	Actinobacteria	MB-A2-108	0319-7L14	unidentified	unidentified	unidentified
483	Proteobacteria	α-proteobacteria	Rhizobi	Hyphomicrobi	unidentified	unidentified

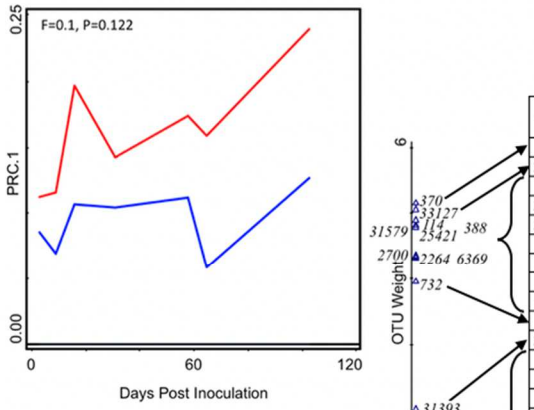
Figure 8: Principal response curve coefficients (PRC) of Year 1 16S sequences for Field A. Curves represent deviation between a compost treatment (baseline NC (black), DC (blue), PL (red)). Curves represent deviation between a compost treatment (baseline NC (black), DC (blue), PL (red)). Missing taxonomic information was not included for the OTU. Monte Carlo permutation tests permutated time to compute statistical significance.





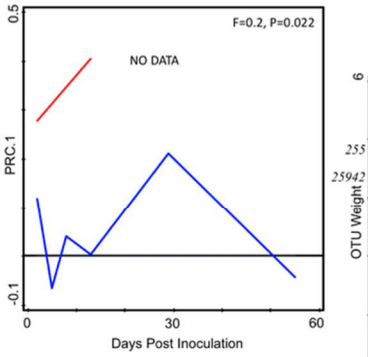
OTU#	Phylum	Class	Order (-ales)	Family (-aceae)	Genus	Species
262	Bacteroidetes	Sphingobacteriia	Sphingobacteri	Sphingobacteri	Pedobacter	unidentified
326	Bacteroidetes	Flavobacteriia	Flavobacteri	Flavobacteri	Flavobacterium	succinicans
34390	Bacteroidetes	Flavobacteriia	Flavobacteri	Flavobacteri	Flavobacterium	succinicans
5358	Bacteroidetes	Sphingobacteriia	Sphingobacteri	Sphingobacteri	Pedobacter	unidentified
284	Bacteroidetes	Sphingobacteriia	Sphingobacteri	Sphingobacteri	Pedobacter	unidentified
698	Bacteroidetes	Flavobacteriia	Flavobacteri	Weeksell	Chryseobacterium	unidentified
33438	Bacteroidetes	Sphingobacteriia	Sphingobacteri	Sphingobacteri	unidentified	unidentified
14533	Proteobacteria	ß-proteobacteria	Burkholderi	Oxalobacter	unidentified	unidentified
4585	Proteobacteria	ß-proteobacteria	Burkholderi	Oxalobacter	Janthinobacterium	unidentified
34099	Bacteroidetes	Flavobacteriia	Flavobacteri	Flavobacteri	Flavobacterium	succinicans
35458	Proteobacteria	γ-proteobacteria	Xanthomonad	Xanthomonad	Stenotrophomonas	unidentified
29153	Bacteroidetes	Sphingobacteriia	Sphingobacteri	Sphingobacteri	Pedobacter	unidentified
2858	Bacteroidetes	Flavobacteriia	Flavobacteri	Flavobacteri	Flavobacterium	succinicans
300	Proteobacteria	γ-proteobacteria	Xanthomonad	Xanthomonad	Stenotrophomonas	maltophilia
497	Bacteroidetes	Sphingobacteriia	Sphingobacteri	Sphingobacteri	Sphingobacterium	multivorum
7799	Bacteroidetes	Saprospirae	Saprospir	Chitinophag	unidentified	unidentified
25388	Bacteroidetes	Cytophagia	Cytophag	Cytophag	Dyadobacter	unidentified
2798	Bacteroidetes	Sphingobacteriia	Sphingobacteri	unidentified	unidentified	unidentified
1691	Planctomycetes	Phycisphaerae	WD2101	unidentified	unidentified	unidentified
14	Proteobacteria	α-proteobacteria	Sphingomonad	Sphingomonad	Kaistobacter	unidentified

Figure 9: Principal response curve coefficients (PRC) of Year 2 16S sequences for Field A. Curves represent deviation between a compost treatment (baseline NC (black), DC (blue), PL (red)). Curves represent deviation between a compost treatment (baseline NC (black), DC (blue), PL (red)). Missing taxonomic information was not included for the OTU. Monte Carlo permutation tests permutated time to compute statistical significance.



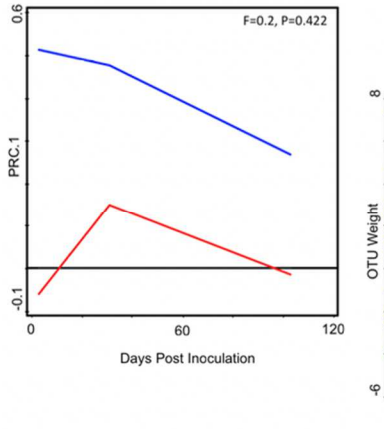
OTU#	Phylum	Class	Order (-ales)	Family (-aceae)	Genus
370	Acidobacteria	Chloracidobacteria	RB41	unidentified	unidentified
33127	Proteobacteria	α-proteobacteria	Sphingomonad	Sphingomonad	Sphingomonas
114	Acidobacteria	Chloracidobacteria	RB41	Ellin6075	unidentified
31579	Proteobacteria	α-proteobacteria	Sphingomonad	Sphingomonad	Sphingomonas
25421	Acidobacteria	Chloracidobacteria	RB41	Ellin6075	unidentified
388	Acidobacteria	Chloracidobacteria	RB41	Ellin6075	unidentified
2700	Verrucomicrobia	Opitutae	Opitut	Opitut	Opitutus
2264	Planctomycetes	Planctomycetia	Pirellul	Pirellul	unidentified
6369	Proteobacteria	α-proteobacteria	Rhizobi	Bradyrhizobi	Balneimonas
732	Gemmatimonadetes	Gemm-1	unidentified	unidentified	unidentified
31393	Acidobacteria	unidentified	unidentified	unidentified	unidentified
164	Proteobacteria	ß-proteobacteria	Ellin6067	unidentified	unidentified
888	Proteobacteria	Δ-proteobacteria	unidentified	unidentified	unidentified
264	Proteobacteria	α-proteobacteria	Rhodospirill	Rhodospirill	unidentified
30437	Bacteroidetes	Cytophagia	Cytophag	Cytophag	unidentified
299	Acidobacteria	unidentified	unidentified	unidentified	unidentified
1184	Verrucomicrobia	Spartobacteria	Chthoniobacter	Chthoniobacter	OR-59
815	Proteobacteria	Δ-proteobacteria	Syntrophobacter	Syntrophobacter	unidentified
181	unidentified	unidentified	unidentified	unidentified	unidentified
149	WS3	PRR-12	Sediment-1	unidentified	unidentified

Figure 10: Principal response curve coefficients (PRC) of Year 1 16S sequences for Field B. Curves represent deviation between a compost treatment (baseline NC (black), DC (blue), PL (red)). Curves represent deviation between a compost treatment (baseline NC (black), DC (blue), PL (red)). Missing taxonomic information was not included for the OTU. Monte Carlo permutation tests permutated time to compute statistical significance.



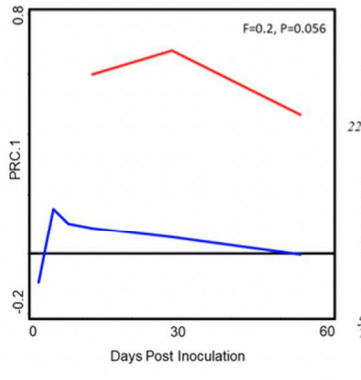
OTU#	Phylum	Class	Order (-ales)	Family (-aceae)	Genus	Species
7864	Proteobacteria	ß-proteobacteria	Burkholderi	Oxalobacter	unidentified	unidentified
255	Bacteroidetes	Sphingobacteriia	Sphingobacteri	Sphingobacteri	unidentified	unidentified
25329	Proteobacteria	ß-proteobacteria	Burkholderi	Oxalobacter	Massilia	timonae
284	Bacteroidetes	Sphingobacteriia	Sphingobacteri	Sphingobacteri	Pedobacter	unidentified
5358	Bacteroidetes	Sphingobacteriia	Sphingobacteri	Sphingobacteri	Pedobacter	unidentified
14533	Proteobacteria	ß-proteobacteria	Burkholderi	Oxalobacter	unidentified	unidentified
5605	Bacteroidetes	Sphingobacteriia	Sphingobacteri	Sphingobacteri	unidentified	unidentified
25942	Bacteroidetes	Sphingobacteriia	Sphingobacteri	Sphingobacteri	Mucilaginibacter	unidentified
354	Proteobacteria	α-proteobacteria	Rhizobi	Bradyrhizobi	Balneimonas	unidentified
818	Gemmatimonadetes	Gemmatimonadetes	Ellin5290	unidentified	unidentified	unidentified
31969	Proteobacteria	γ-proteobacteria	Enterobacteri	Enterobacteri	unidentified	unidentified
3708	Planctomycetes	Planctomycetia	Pirellul	Pirellul	unidentified	unidentified
2478	Actinobacteria	MB-A2-108	unidentified	unidentified	unidentified	unidentified
28585	Proteobacteria	α-proteobacteria	unidentified	unidentified	unidentified	unidentified
13	Crenarchaeota	Thaumarchaeota	Nitrososphaer	Nitrososphaer	Candidatus	nitrososphaera
31530	Proteobacteria	γ-proteobacteria	Xanthomonad	Sinobacter	unidentified	unidentified
301	Proteobacteria	Δ-proteobacteria	Syntrophobacter	Syntrophobacter	unidentified	unidentified
467	Gemmatimonadetes	Gemm-1	unidentified	unidentified	unidentified	unidentified
365	Proteobacteria	Δ-proteobacteria	Myxococc	unidentified	unidentified	unidentified
489	Actinobacteria	Acidimicrobiia	Acidimicrobi	unidentified	unidentified	unidentified

Figure 11: Principal response curve coefficients (PRC) of Year 2 16S sequences for Field B. Curves represent deviation between a compost treatment (baseline NC (black), DC (blue), PL (red)). Curves represent deviation between a compost treatment (baseline NC (black), DC (blue), PL (red)). Missing taxonomic information was not included for the OTU. Monte Carlo permutation tests permutated time to compute statistical significance.



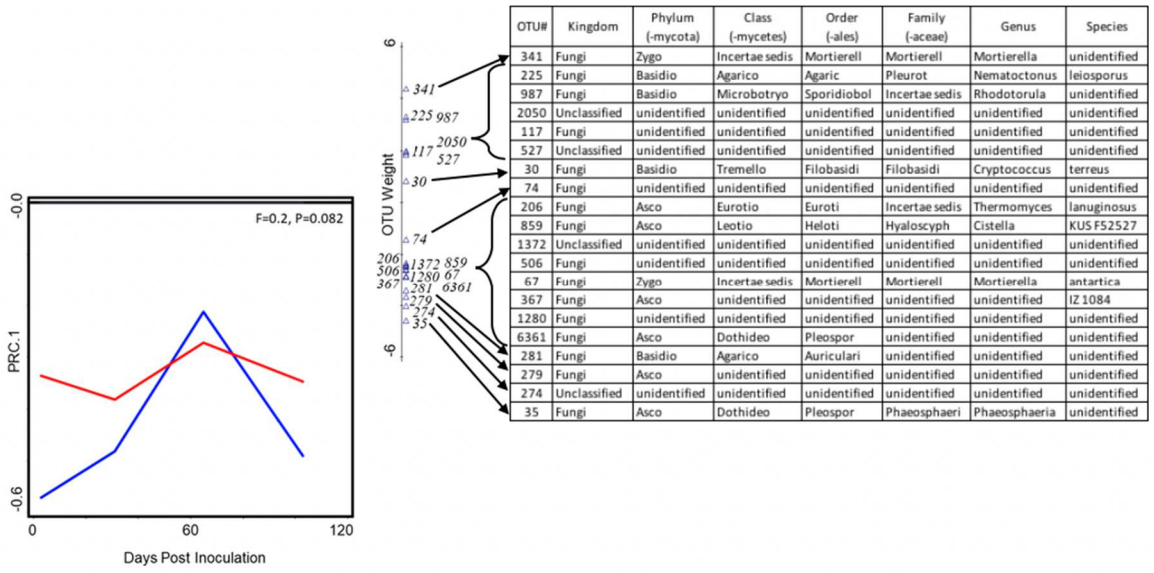
OTU#	Kingdom	Phylum (-mycota)	Class (-mycetes)	Order (-ales)	Family (-aceae)	Genus	Species
13	Fungi	Basidio	Agarico	Agaric	Bolbiti	Conocybe	apala
92	Fungi	Basidio	Agarico	Agaric	Psathyrell	Coprinellus	bisporus
714	Fungi	Asco	Dothideo	Pleospor	unidentified	unidentified	unidentified
1187	Fungi	Asco	unidentified	unidentified	unidentified	unidentified	unidentified
4389	Fungi	Zygo	Incertae sedis	Mortierell	Mortierell	Mortierella	camargensis
435	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
373	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
2356	Fungi	Asco	Dothideo	Pleospor	unidentified	unidentified	unidentified
10605	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
99	Protista	Cercozoa	unidentified	unidentified	unidentified	unidentified	unidentified
860	Fungi	Basidio	Agarico	Agaric	Entolomat	Entoloma	unidentified
1126	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	uncultured fungus
197	Fungi	Asco	Sordario	Microasc	Microasc	Scedosporium	prolificans
1119	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
21550	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
753	Fungi	Basidio	Agarico	Agaric	Entolomat	Entoloma	unidentified
832	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
61	Fungi	Basidio	Agarico	Agaric	Agaric	Disciseda	candida
1095	Fungi	Zygo	Incertae sedis	Mortierell	Mortierell	unidentified	unidentified
14	Fungi	Zygo	Incertae sedis	Mortierell	Mortierell	Mortierella	unidentified

Figure 12: Principal response curve coefficients (PRC) of Year 1 ITS sequences for Field A. Curves represent deviation between a compost treatment (baseline NC (black), DC (blue), PL (red)). Curves represent deviation between a compost treatment (baseline NC (black), DC (blue), PL (red)). Missing taxonomic information was not included for the OTU. Monte Carlo permutation tests permutated time to compute statistical significance.



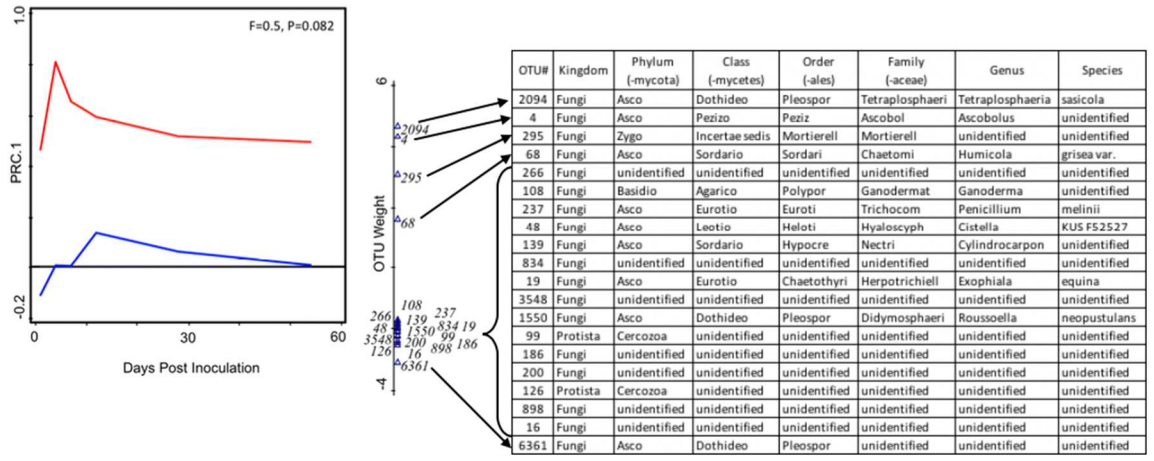
OTU#	Kingdom	Phylum (-mycota)	Class (-mycetes)	Order (-ales)	Family (-aceae)	Genus	Species
17	Fungi	Asco	Pezizo	Peziz	Ascobol	Ascobolus	unidentified
2094	Fungi	Asco	Dothideo	Pleospor	Tetraplosphaeri	Tetraplosphaeria	sasicola
22104	Fungi	Asco	Sordario	Sordari	Lasiosphaeri	unidentified	uncultured Cercophora
122	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
306	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
225	Fungi	Basidio	Agarico	Agaric	Pleurot	Nematoctonus	leiosporus
68	Fungi	Asco	Sordario	Sordari	Chaetomi	Humicola	grisea var.
555	Fungi	Asco	Sordario	Sordari	Lasiosphaeri	unidentified	uncultured Cercophora
4	Fungi	Asco	Pezizo	Peziz	Ascobol	Ascobolus	unidentified
2012	Fungi	Asco	Dothideo	Pleospor	Phaeosphaeri	Phaeosphaeria	SS 1624
3913	Protista	Cercozoa	unidentified	unidentified	unidentified	unidentified	unidentified
1889	Fungi	Asco	unidentified	unidentified	unidentified	unidentified	r238
19	Fungi	Asco	Eurotio	Chaetothyri	Herpotrichiell	Exophiala	equina
1667	Fungi	Asco	Dothideo	Capnodi	Mycosphaerell	Septoria	unidentified
1031	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
48	Fungi	Asco	Leotio	Heloti	Hyaloscyph	Cistella	KUS F52527
524	Fungi	Asco	Sordario	Hypocre	Hypocre	Trichoderma	spirale
1639	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
120	Fungi	Asco	unidentified	unidentified	unidentified	unidentified	unidentified
773	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified

Figure 13: Principal response curve coefficients (PRC) of Year 2 ITS sequences for Field A. Curves represent deviation between a compost treatment (baseline NC (black), DC (blue), PL (red)). Curves represent deviation between a compost treatment (baseline NC (black), DC (blue), PL (red)). Missing taxonomic information was not included for the OTU. Monte Carlo permutation tests permutated time to compute statistical significance.



OTU#	Kingdom	Phylum (-mycota)	Class (-mycetes)	Order (-ales)	Family (-aceae)	Genus	Species
341	Fungi	Zygo	Incertae sedis	Mortierell	Mortierell	Mortierella	unidentified
225	Fungi	Basidio	Agarico	Agaric	Pleurot	Nematoctonus	leiosporus
987	Fungi	Basidio	Microbotryo	Sporidiobol	Incertae sedis	Rhodotorula	unidentified
2050	Unclassified	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
117	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
527	Unclassified	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
30	Fungi	Basidio	Tremello	Filobasidi	Filobasidi	Cryptococcus	terreus
74	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
206	Fungi	Asco	Eurotio	Euroti	Incertae sedis	Thermomyces	lanuginosus
859	Fungi	Asco	Leotio	Heloti	Hyaloscyph	Cistella	KUS F52527
1372	Unclassified	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
506	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
67	Fungi	Zygo	Incertae sedis	Mortierell	Mortierell	Mortierella	antartica
367	Fungi	Asco	unidentified	unidentified	unidentified	unidentified	IZ 1084
1280	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
6361	Fungi	Asco	Dothideo	Pleospor	unidentified	unidentified	unidentified
281	Fungi	Basidio	Agarico	Auriculari	unidentified	unidentified	unidentified
279	Fungi	Asco	unidentified	unidentified	unidentified	unidentified	unidentified
274	Unclassified	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
35	Fungi	Asco	Dothideo	Pleospor	Phaeosphaeri	Phaeosphaeria	unidentified

Figure 14: Principal response curve coefficients (PRC) of Year 1 ITS sequences for Field B. Curves represent deviation between a compost treatment (baseline NC (black), DC (blue), PL (red)). Curves represent deviation between a compost treatment (baseline NC (black), DC (blue), PL (red)). Missing taxonomic information was not included for the OTU. Monte Carlo permutation tests permutated time to compute statistical significance.



OTU#	Kingdom	Phylum (-mycota)	Class (-mycetes)	Order (-ales)	Family (-aceae)	Genus	Species
2094	Fungi	Asco	Dothideo	Pleospor	Tetraplosphaeri	Tetraplosphaeria	sasicola
4	Fungi	Asco	Pezizo	Peziz	Ascobol	Ascobolus	unidentified
295	Fungi	Zygo	Incertae sedis	Mortierell	Mortierell	unidentified	unidentified
68	Fungi	Asco	Sordario	Sordari	Chaetomi	Humicola	grisea var.
266	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
108	Fungi	Basidio	Agarico	Polypor	Ganodermat	Ganoderma	unidentified
237	Fungi	Asco	Eurotio	Euroti	Trichocom	Penicillium	melinii
48	Fungi	Asco	Leotio	Heloti	Hyaloscyph	Cistella	KUS F52527
139	Fungi	Asco	Sordario	Hypocre	Nectri	Cylindrocarpon	unidentified
834	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
19	Fungi	Asco	Eurotio	Chaetothyri	Herpotrichiell	Exophiala	equina
3548	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
1550	Fungi	Asco	Dothideo	Pleospor	Didymosphaeri	Roussoella	neopustulans
99	Protista	Cercozoa	unidentified	unidentified	unidentified	unidentified	unidentified
186	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
200	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
126	Protista	Cercozoa	unidentified	unidentified	unidentified	unidentified	unidentified
898	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
16	Fungi	unidentified	unidentified	unidentified	unidentified	unidentified	unidentified
6361	Fungi	Asco	Dothideo	Pleospor	unidentified	unidentified	unidentified

Figure 15: Principal response curve coefficients (PRC) of Year 2 ITS sequences for Field B. Curves represent deviation between a compost treatment (baseline NC (black), DC (blue), PL (red)). Curves represent deviation between a compost treatment (baseline NC (black), DC (blue), PL (red)). Missing taxonomic information was not included for the OTU. Monte Carlo permutation tests permutated time to compute statistical significance.

References

Anderson, M., Jaykus, L. A., Beaulieu, S., & Dennis, S. (2011). Pathogen-produce pair attribution risk ranking tool to prioritize fresh produce commodity and pathogen combinations for further evaluation (P3ARRT). *Food Control*, *22*(12), 1865–1872. http://doi.org/10.1016/j.foodcont.2011.04.028

Barnard, R. L., Osborne, C. A., & Firestone, M. K. (2013). Responses of soil bacterial and fungal communities to extreme desiccation and rewetting. *ISME Journal*, *7*(11), 2229–2241. http://doi.org/10.1038/ismej.2013.104

Beuchat, L. R., & Brackett, R. E. (1991). Behavior of *Listeria monocytogenes* inoculated into raw tomatoes and processed tomato products. *Applied and Environmental Microbiology*, *57*(5), 1367–1371

Brochier, V., Gourland, P., Kallassy, M., Poitrenaud, M., & Houot, S. (2012). Occurrence of pathogens in soils and plants in a long-term field study regularly amended with different composts and manure. *Agriculture, Ecosystems and Environment*, *160*, 91–98. http://doi.org/10.1016/j.agee.2011.05.021

Cebrián, G., Condón, S., & Mañas, P. (2017). Physiology of the Inactivation of Vegetative Bacteria by Thermal Treatments: Mode of Action, Influence of Environmental Factors and Inactivation Kinetics. *Foods*, *6*(12), 107. http://doi.org/10.3390/foods6120107

Cooley, M., Carychao, D., Crawford-Miksza, L., Jay, M. T., Myers, C., Rose, C., Keys C., Farrar J., & Mandrell, R. E. (2007). Incidence and tracking of *Escherichia coli* O157:H7 in a major produce production region in California. *PLoS ONE*, *2*(11). http://doi.org/10.1371/journal.pone.0001159

Cutler, A.J., Neher, D.A., Weicht, T.R., Sharma, M., and Millner, P. (2018) *Escherichia coli* survival and microbial community dynamics in compost-amended soils. *Microbial Ecology* MECO-D-18-00672 (submitted 10/23/18)

D'Amico, D. J., & Donnelly, C. W. (2009). Detection, isolation, and incidence of *Listeria* spp. in small-scale artisan cheese processing facilities: a methods comparison. *Journal of Food Protection*, *72*(12), 2499–2507

DeLaune P.B., Moore P.A., Daniel T.C., Lemunyon J.L. (2004). Effect of chemical and microbial amendments on ammonia voltalization from composting poultry litter. *J Environ Qual* 33:728-734

Edgar, R. C. (2013). UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nature Methods*, *10*(10), 996–998. http://doi.org/10.1038/nmeth.2604

Emerson, J. B., Keady, P. B., Brewer, T. E., Clements, N., Morgan, E. E., Awerbuch, J., Miller, S.L., and Fierer, N. (2015). Impacts of flood damage on airborne bacteria and fungi in homes after the 2013 Colorado front range flood. *Environmental Science and Technology*, *49*(5), 2675–2684. http://doi.org/10.1021/es503845j

Franz, E., Semenov, A. V., Termorshuizen, A. J., De Vos, O. J., Bokhorst, J. G., & Van Bruggen, A. H. C. (2008). Manure-amended soil characteristics affecting the survival of *E. coli* O157:H7 in 36 Dutch soils. *Environmental Microbiology*, *10*(2), 313–327. http://doi.org/10.1111/j.1462-2920.2007.01453.x

Fremaux, B., Prigent-Combaret, C., Delignette-Muller, M. L., Mallen, B., Dothal, M., Gleizal, A., & Vernozy-Rozand, C. (2008). Persistence of Shiga toxin-producing *Escherichia coli* O26 in various manure-amended soil types. *Journal of Applied Microbiology*, *104*(1), 296–304. http://doi.org/10.1111/j.1365-2672.2007.03532.x

Fremaux, B., Prigent-Combaret, C., & Vernozy-Rozand, C. (2008). Long-term survival of Shiga toxin-producing *Escherichia coli* in cattle effluents and environment: An updated review. *Veterinary Microbiology*, *132*(1–2), 1–18. http://doi.org/10.1016/j.vetmic.2008.05.015.

Grace, J. B., Anderson, T. M., Olff, H., & Scheiner, S. M. (2010). On the specification of structural equation models for ecological systems. *Ecological Monographs*, *80*(1), 67–87. http://doi.org/10.1890/09-0464.1

Gutiérrez-Rodríguez, E., Gundersen, A., Sbodio, A. O., & Suslow, T. V. (2012). Variable agronomic practices, cultivar, strain source and initial contamination dose differentially affect survival of *Escherichia coli* on spinach. *Journal of Applied Microbiology*, *112*(1), 109–118. http://doi.org/10.1111/j.1365-2672.2011.05184.x

Harris, L. J., Farber, J. N., Beuchat, L. R., Parish, M. E., Suslow, T. V., Garrett, E. H., & Busta, F. F. (2003). Outbreaks associated with fresh produce: incidence, growth and survival of pathogens in fresh and fresh-cut produce. *Comprehensive Reviews in Food Science and Food Safety*, *2*(s1), 78–141. http://doi.org/10.1111/j.1541-4337.2003.tb00031.x

Heiman, K. E., Mody, R. K., Johnson, S. D., Griffin, P. M., & Gould, L. H. (2015). *Escherichia coli* O157 Outbreaks in the United States , 2003 – 2012. *Emerg Infect Dis*, *21*(8), 1293–1301. http://doi.org/10.3201/eid2108.141364

Hu, L., Robert, C. A. M., Cadot, S., Zhang, X., Ye, M., Li, B., Manzo, D., Chervet, N., Steinger., Van Der H., Marcel G.A., Schlaeppi, K., Erb M. (2018). Root exudate metabolites drive plant-soil feedbacks on growth and defense by shaping the rhizosphere microbiota. *Nature Communications*, *9*(1), 1–13. https://doi.org/10.1038/s41467-018-05122-7

Hutchison, M. L., Walters, L. D., Moore, A., Crookes, K. M., & Avery, S. M. (2004). Effect of Length of Time before Incorporation on Survival of Pathogenic Bacteria Present in Livestock Wastes Applied to Agricultural Soil Effect of Length of Time before Incorporation on Survival of Pathogenic Bacteria Present in Livestock Wastes Applied. *Applied and Environmental Microbiology*, *70*(9), 5111–5118. http://doi.org/10.1128/AEM.70.9.5111

Ingram, D. T., Patel, J., & Sharma, M. (2011). Effect of Repeated Irrigation with Water Containing Varying Levels of Total Organic Carbon on the Persistence of *Escherichia coli* O157:H7 on Baby Spinach. *Journal of Food Protection*, *74*(5), 709–717. http://doi.org/10.4315/0362-028X.JFP-10-426

Islam, M., Morgan, J., Doyle, M. P., & Jiang, X. (2004). Fate of *Escherichia coli* O157:H7 in Manure Compost–Amended Soil and on Carrots and Onions Grown in an Environmentally Controlled Growth Chamber. *Journal of Food Protection*, *67*(3), 574–578. http://doi.org/10.4315/0362-028X-67.3.574

Islam, M., Morgan, J., Doyle, M. P., Phatak, S. C., Millner, P., & Jiang, X. (2004). Persistence of *Salmonella enterica* Serovar Typhimurium on Lettuce and Parsley and in Soils on Which They Were Grown in Fields Treated with Contaminated Manure Composts or Irrigation Water. *Foodborne Pathogens and Disease*, *1*(1), 27–35. http://doi.org/10.1089/153531404772914437

Jamieson, R. C., Gordon, R. J., Sharples, K. E., Stratton, G. W., & Madani, A. (2002). Movement and persistence of fecal bacteria in agricultural soils and subsurface drainage water: A review. *Canadian Biosystems Engineering*, *44*, 1.1-1.9

Jiang, X., Morgan, J., & Doyle, M. P. (2002). Fate of *Escherichia coli* O157: H7 in Manure-Amended Soil. *Applied and Environmental Microbiology*, *68*(5), 2605–2609. http://doi.org/10.1128/AEM.68.5.2605

Kelleher, B. P., Leahy, J. J., Henihan, a M., O'Dwyer, T. F., Sutton, D., & Leahy, M. J. (2002). Advances in poultry litter disposal technology--a review. *Bioresource Technology*, *83*(1), 27–36. http://doi.org/10.1016/S0960-8524(01)00133-X.

Kõljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AFS, Bahram M, Bates ST, Bruns TD, Bengtsson-Palme J, Callaghan TM, Douglas B (2013). Towards a unified paradigm for sequence-based identification of fungi. *Mol Ecol* 22: 5271−5277

Lau, M. M., & Ingham, S. C. (2001). Survival of faecal indicator bacteria in bovine manure incorporated into soil. *Letters in Applied Microbiology*, *33*(2), 131–136. http://doi.org/10.1046/j.1472-765X.2001.00962.x

Lauber, C. L., Hamady, M., Knight, R., & Fierer, N. (2009). Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Applied and Environmental Microbiology*, *75*(15), 5111–5120. http://doi.org/10.1128/AEM.00335-09

Lekkas, P. (2016). *The Microbial Ecology Of Listeria monocytogenes As Impacted By Three Environments: A Cheese Microbial Community ; A Farm Environment ; And A Soil Microbial Community; Chapter 4:* Fate Of *Escherichia coli* And *Listeria* Species In Dairy Manure Amendments Applied In Soils In The Northeastern United States. Thesis

Locatelli A., Spor A., Jolivet C., Piveteau P., & Hartmann A. (2013). Biotic and Abiotic Soil Properties Influence Survival of *Listeria monocytogenes* in Soil. *PLOS ONE* 8(10): e75969. https://doi.org/10.1371/journal.pone.0075969

Sharma M. and Reynnells R.. (2016). Importance of Soil Amendments: Survival of Bacterial Pathogens in Manure and Compost Used as Organic Fertilizers. *Microbiology Spectrum*, *4*(4), 1–13. http://doi.org/doi:10.1128/microbiolspec.PFS-0010-2015

Markland, S. M., Shortlidge, K. L., Hoover, D. G., Yaron, S., Patel, J., Singh, A., Sharma, M., Kniel, K. E. (2013). Survival of Pathogenic *Escherichia coli* on Basil, Lettuce, and Spinach. *Zoonoses and Public Health*, *60*(8), 563–571. http://doi.org/10.1111/zph.12033

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., Desantis, T. Z., Probst, A., Andersen G.L., Knight R., & Hugenholtz, P. (2011). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME Journal*, *6*(3), 610–618. http://doi.org/10.1038/ismej.2011.139

Moorhead, D. L., Rinkes, Z. L., Sinsabaugh, R. L., & Weintraub, M. N. (2013). Dynamic relationships between microbial biomass, respiration, inorganic nutrients and enzyme activities: Informing enzyme-based decomposition models. *Frontiers in Microbiology*, *4*(AUG), 1–12. http://doi.org/10.3389/fmicb.2013.00223

Moorhead, D. L., Sinsabaugh, R. L., Hill, B. H., & Weintraub, M. N. (2016). Vector analysis of ecoenzyme activities reveal constraints on coupled C, N and P dynamics. *Soil Biology and Biochemistry*, *93*, 1–7. http://doi.org/10.1016/j.soilbio.2015.10.019

Moyne, A. L., Sudarshana, M. R., Blessington, T., Koike, S. T., Cahn, M. D., & Harris, L. J. (2011). Fate of *Escherichia coli* O157:H7 in field-inoculated lettuce. *Food Microbiology*, *28*(8), 1417–1425. http://doi.org/10.1016/j.fm.2011.02.001

Natvig, E. E., Ingham, S. C., Ingham, B. H., Cooperband, L. R., & Roper, T. R. (2002). *Salmonella* enterica serovar typhimurium and *Escherichia coli* contamination of root

and leaf vegetables grown in soils with incorporated bovine manure. *Applied and Environmental Microbiology*, *68*(6), 2737–2744. http://doi.org/10.1128/AEM.68.6.2737-2744.2002

Neher, D. A., Weicht, T. R., Bates, S. T., Leff, J. W., & Fierer, N. (2013). Changes in bacterial and fungal communities across compost recipes, preparation methods, and composting times. *PLoS ONE*, *8*(11). http://doi.org/10.1371/journal.pone.0079512.

Neher, D. A., Wu, J., Barbercheck, M. E., & Anas, O. (2005). Ecosystem type affects interpretation of soil nematode community measures. *Applied Soil Ecology*, *30*(1), 47–64. http://doi.org/10.1016/j.apsoil.2005.01.002

Nightingale, K. K., Schukken, Y. H., Nightingale, C. R., Fortes, E. D., Ho, A. J., Her, Z., McDonough P.L., Wiedmann, M. (2004). Ecology and Transmission of *Listeria monocytogenes* Infecting Ruminants and in the Farm Environment, *70*(8), 4458–4467. *Appl Environ Microbiol*. http://doi.org/10.1128/AEM.70.8.4458

Olaimat, A. N., & Holley, R. A. (2012). Factors influencing the microbial safety of fresh produce: A review. *Food Microbiology*, *32*(1), 1–19. http://doi.org/10.1016/j.fm.2012.04.016

Painter, J. A., Hoekstra, R. M., Ayers, T., Tauxe, R. V., Braden, C. R., Angulo, F. J., & Griffin, P. M. (2013). Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. *Emerging Infectious Diseases*, *19*(3), 407–415. http://doi.org/10.3201/eid1903.111866

Park, S., Szonyi, B., Gautam, R., Nightingale, K., Anciso, J., & Ivanek, R. (2012). Risk Factors for Microbial Contamination in Fruits and Vegetables at the Preharvest Level: A Systematic Review. *Journal of Food Protection*, *75*(11), 2055–2081. http://doi.org/10.4315/0362-028X.JFP-12-160

Paul E.A. (2015) Soil microbiology, ecology, and biochemistry 4th edn. Elsevier, New York.

Peters, J. B. (2003). Recommended Methods of Manure Analysis. *Soils.Wisc.Edu*, 1–58. http://doi.org/Recommended Methods of Manure Anaysis (A3769

Pugliese, M., Liu, B. P., Gullino, M. L., & Garibaldi, A. (2008). Selection of antagonists from compost to control soil-borne pathogens. *Journal of Plant Diseases and Protection*, *115*(5), 220–228.

Reitzer L. (2003). Nitrogen Assimilation and Global Regulation in *Escherichia coli*. *Annual Review Microbiology* 57: 155-176

Reynnells, R., Ingram, D. T., Roberts, C., Stonebraker, R., Handy, E. T., Felton, G., Vinyard B.T., Millner P.D. & Sharma, M. (2014). Comparison of U.S. Environmental Protection Agency and U.S. Composting Council Microbial Detection Methods in Finished Compost and Regrowth Potential of *Salmonella* spp. and *Escherichia coli* O157:H7 in Finished Compost. *Foodborne Pathogens and Disease*, *11*(7), 555–567. http://doi.org/10.1089/fpd.2013.1698

Santorum, P., García, R., López, V., & Martínez-Suárez, J. V. (2012). Review. Dairy farm management and production practices associated with the presence of *Listeria monocytogenes* in raw milk and beef. *Spanish Journal of Agricultural Research*, *10*(2), 360. http://doi.org/10.5424/sjar/2012102-314-11

Scharff, R. L. (2012). Economic Burden from Health Losses Due to Foodborne Illness in the United States. *Journal of Food Protection*, *75*(1), 123–131. http://doi.org/10.4315/0362-028X.JFP-11-058

Semenov, A. V., Van Bruggen, A. H. C., Van Overbeek, L., Termorshuizen, A. J., & Semenov, A. M. (2007). Influence of temperature fluctuations on *Escherichia coli* O157:H7 and *Salmonella* enterica serovar Typhimurium in cow manure. *FEMS Microbiology Ecology*, *60*(3), 419–428. http://doi.org/10.1111/j.1574-6941.2007.00306.x

Sharma, M., Millner, P. D., Hashem, F., Camp, M., Whyte, C., Graham, L., & Cotton, C. P. (2016). Survival and Persistence of Nonpathogenic *Escherichia coli* and Attenuated *Escherichia coli* O157:H7 in Soils Amended with Animal Manure in a Greenhouse Environment. *Journal of Food Protection*, *79*(6), 913–921. http://doi.org/10.4315/0362-028X.JFP-15-421

Singh, R., Jiang, X., & Luo, F. (2010). Thermal inactivation of heat-shocked *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in dairy compost. *Journal of Food Protection*, *73*(9), 1633–1640.

Sinsabaugh, R. L., & Follstad Shah, J. J. (2012). Ecoenzymatic Stoichiometry and Ecological Theory. *Annual Review of Ecology, Evolution, and Systematics*, *43*(1), 313–343. http://doi.org/10.1146/annurev-ecolsys-071112-124414

Tiedje, J.M., Sexstone, A.J., Parkin, T.B., Revsbech N. P. (1984). *Plant Soil*. 76: 197. https://doi.org/10.1007/BF02205580

Tomás-Callejas, A., López-Velasco, G., Camacho, A. B., Artés, F., Artés-Hernández, F., & Suslow, T. V. (2011). Survival and distribution of *Escherichia coli* on diverse fresh-cut baby leafy greens under preharvest through postharvest conditions. *International Journal of Food Microbiology*, *151*(2), 216–222. http://doi.org/10.1016/j.ijfoodmicro.2011.08.027

Trmčić, A., Ralyea, R., Meunier-Goddik, L., Donnelly, C., Glass, K., D'Amico, D., Meredith E., Kehler M., Tranchina N., McCue C., & Wiedmann, M. (2017). Consensus categorization of cheese based on water activity and pH—A rational approach to systemizing cheese diversity. *Journal of Dairy Science*, *100*(1), 841–847. http://doi.org/10.3168/jds.2016-11621

U.S. FDA. (n.d.). FDA Fact Sheet: Produce Safety Rule. Retrieved from http://www.fda.gov/ForConsumers/ConsumerUpdates/ucm107358.htm

U.S Food and Drug Administration (FDA). (2018a). Code of Federal Regulation Title 21. Current Good Manufacturing Practice in Maufacturing, Packing, or Holding Human Food. www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?CFRPart=112. Accessed November 3, 2018.

U.S. FDA (2018b) FSMA Final Rule on Produce Safety. https://www.fda.gov/food/guidanceregulation/fsma/ucm334114.htm. Accessed Nov 4, 2018

U.S FDA (2016). FSMA Rules and Guidance for Industry. http://www.fda.gov/Food/GuidanceRegulation/FSMA/ucm334552.htm. Accessed March 20, 2016

U.S. FDA. (2014). FDA at a Glance Key Revisions on Produce Safety, 1–2.

U.S. FDA. (2013). Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption; Proposed Rule. *2013*, 1–548

U.S. Food and Drug Administration (FDA). (2011). FSMA Facts: background on the FDA food safety modernization act (FMSA). *FDA Food Safety Modernization Act*, 1–3. Retrieved from http://www.fda.gov/Food/GuidanceRegulation/FSMA/ucm239907.htm Accessed November 4, 2018.

Van Den Brink, P. J., & Ter Braak, C. J. F. (1999). Principal response curves: Analysis of time-dependent multivariate responses of biological community to stress. *Environmental Toxicology and Chemistry*, *18*(2), 138–148. http://doi.org/10.1897/1551-5028(1999)018<0138:PRCAOT>2.3.CO;2

Van den Brink, P. J., Van den Brink, N. W., & Ter Braak, C. J. F. (2003). Multivariate Analysis of Ecotoxicological Data Using Ordination: Demonstrations of Utility on the Basis of Various Examples. *Australasian Journal of Ecotoxicology*, *9*, 141–156. Retrieved from http://www.biometris.wur.nl/NR/rdonlyres/71EBBDE7-DE7B-4956-9BEA-2B807C34A8EF/47864/terBraak2003VandenBrink2Braak_AJE.pdf

Van Elsas, J. D., Semenov, A. V., Costa, R., & Trevors, J. T. (2011). Survival of *Escherichia coli* in the environment: Fundamental and public health aspects. *ISME Journal*, *5*(2), 173–183. http://doi.org/10.1038/ismej.2010.80

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261–5267

Whalley, W. R., Ober, E. S., & Jenkins, M. (2013). Measurement of the matric potential of soil water in the rhizosphere. *Journal of Experimental Botany*, *64*(13), 3951–3963. http://doi.org/10.1093/jxb/ert044

Yeoh Y.K., Sekiguchi Y., Parks D.H., & Hugenholtz P. Comparative Genomics of Candidate Phylum TM6 Suggests That Parasitism Is Widespread and Ancestral in This Lineage. Mol. Biol.Evol.33(4):915–927

Zaleski, K. J., Josephson, K. L., Gerba, C. P., & Pepper, I. L. (2005). Potential Regrowth and Recolonization of *Salmonellae* and Indicators in Biosolids and Biosolid-Amended Soil -- Zaleski et al. 71 (7): 3701 -- Applied and Environmental Microbiology, *71*(7), 3701–3708. http://doi.org/10.1128/AEM.71.7.3701

Running Head: Recovery of *Listeria* spp. from Surfaces (Research Paper)

CHAPTER 4: Comparative Recovery of *Listeria* spp. From Dairy Environmental Surfaces Using 3M™ And World Bioproducts© Environmental Swabs and Standard Enrichment and Enumeration Methods

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Abstract

Preventing *Listeria* contamination of artisan cheese requires routine and effective environmental monitoring of product contact surfaces within the production environment. The sensitivity of environmental monitoring methods is essential when testing for the presence of *Listeria* spp. within the processing environment as a way to control the risk of cheese contamination. Four environmental surfaces (dairy brick, stainless steel, plastic, and wood; n=27/surface type at high concentrations; n=405/surface type at low concentrations) were inoculated with *L. innocua* (Green Fluorescent Protein), *L.m.* ATTC® 19115 and *L.m.* 1042B, at high (10^6-10^7 CFU/cm^2) and low (0.01-1 CFU/cm^2) target concentrations. Inoculated surfaces were swabbed with World Bioproducts© EZ Reach™ environmental swabs with HiCap (WBHC) and Dey-Engley (WBDE) neutralizing broths, and 3M™ environmental swabs (3M™) with Dey-Engley neutralizing broth. 3M™ *Listeria* Environmental Plate and Aerobic Plate Count Petrifilm™ enumeration methods and FDA, modified FDA, dual MOPS-BLEB enrichment, and modified USDA enrichment methods were used to compare sensitivity of recovery between environmental swabs. When applied at low concentrations, 3M™, WBDE, and WBHC swabs recovered *Listeria* spp. from 90.9%, 88.4% and 83.2% of plastic, stainless steel, and dairy brick surfaces, respectively, but only 65.7% of wooden surfaces; recovering 14.8%, 77%, and 96.3% at 0.01, 0.1, and 1 CFU/cm^2, respectively ($p<0.05$). Slight differences in recovery (84.8% for WBDE, 78.1% for WBHC, and 80.9% for 3M™) for all surfaces were observed. Variable recovery was influenced by strain, where *L.m.* 1042B was recovered more effectively from wooden surfaces by 3M™, WBDE, and WBHC swabs, followed by *L.m.* 19115, and lastly *L. innocua*. Equivalent performance between swab formats was observed for all tested surfaces except wood, therefore porosity of environmental surfaces should be taken into

consideration when implementing environmental sampling plans.

Introduction

Foods represent a major route of transmission for listeriosis as a result of post-processing contamination, with 99% of illnesses attributed to food products, including ready-to-eat (RTE) foods (Buchanan et al., 2017; Scallan et al., 2011). *Listeria monocytogenes* (*L. monocytogenes*) is the third leading cause of death from a foodborne pathogen (19%), following *Salmonella* spp. (28%) and *Toxoplasma gondii* (24%) (Scallan et al. 2011). Listeriosis, the infection caused by *L. monocytogenes,* is manifest as an invasive disease leading to meningitis, encephalitis, septicemia, neonatal sepsis, and preterm labor. Listeriosis is also manifest as non-invasive infection, which occurs in healthy individuals, with symptoms including febrile gastroenteritis with flu-like symptoms (Scallan et al 2011; Nyarko et al., 2017). Although the incidence of cases of *L. monocytogenes* continues to decline in the U.S., the number of deaths associated with this pathogen of concern continues to increase (CDC, 2017a; Nyachuba & Donnelly, 2007).

L. monocytogenes is widely distributed in dairy farm environments (Nightingale, et al. 2004) and is regularly isolated from dairy processing and cheesemaking environments (Pritchard et al., 1994, Nightingale et al. 2004, D'Amico & Donnelly 2010). 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 (Carpentier, & Cerf, 2011). Recently published studies have shown the contribution of molecular determinants to adaptation and persistence of *Listeria* strains, as well as resistance to

sanitizers (Pan et al. 2006, Buchanan, Gorris et al. 2017, Harter, Wagner et al. 2017, Kremer, Lees et al. 2017).While research has shown that the extent of *Listeria* spp. contamination in farmstead cheese plants is low (D'Amico et al.,. 2008; D'Amico & Donnelly, 2008), some strains of *L. monocytogenes*, including those that may possess increased virulence, have been shown to persist in cheesemaking (D'Amico et al., 2008, D'Amico & Donnelly 2009) and other food processing environments for months or years (Ferreira et al., 2014) and serve as sources of food product contamination (Kovačević et al., 2012; Lahou & Uyttendaele, 2014). Effective environmental monitoring and elimination of *Listeria* spp. within processing plants, including farmstead cheese operations, is thus a key component of a successful *Listeria* control program.

The U.S. Food and Drug Administration (FDA) conducted environmental surveillance of U.S. cheesemakers producing soft cheese (154 plants total, 41 artisan producers) during the years 2010-2011 (Donnelly, 2000). A total of 31% of plants tested had positive environmental findings for *L. monocytogenes*. This unacceptably high incidence shows the need for interventions leading to control and elimination of this dangerous pathogen. In March of 2017, the FDA, CDC and state agencies (CDC, 2017) reported an outbreak of listeriosis caused by consumption of a soft raw milk cheese produced by Vulto Creamery of Walton, New York, which resulted in two deaths and six cases of illness (CDC, 2017). FDA inspections revealed widespread environmental *Listeria* contamination throughout the processing facility (USFDA HHS, 2017). According to the 483 Inspection Report issued by the FDA to Vulto Creamery , 54 out of 198 (27.2%) tested environmental sites were positive for *Listeria* spp.,including floors, drains, exterior surfaces of brine tanks, door handles to the cheese aging room, and

wooden cheese rack dollies (USFDA HHS, 2017). In addition, 10 out of 54 (18.5%) food contact surfaces tested positive for *L. monocytogenes*, including wooden cheese aging boards and cheese brushes.

Food processors could use environmental monitoring programs (EMP) as a verification tool to ensure the control of identified biological hazards from the environment. The artisan cheese industry follows guidelines under 21 CFR 117, Subpart B "Current Good Manufacturing Practices in Manufacturing, Packing, and Holding Human Foods" (USFDA/CFSAN, 2018). There regulations emphasize the importance of cleaning and sanitizing food contact surfaces (USFDA/HHS, 2018).

The FDA has expressed concern over use of wooden shelves as a food contact surface in cheese aging due to their porosity and inability to be effectively cleaned and sanitized (Aviat et al., 2016). The Vulto Creamery listeriosis outbreak investigation cited wooden boards as examples of food contact surface materials whose design did not allow for adequate cleaning and sanitizing as a result of poor maintenance (FDA HHS, 2017; U.S. FDA, 2018).

Dairy processors need assurance that they are using effective methods for environmental sampling, as well as sensitive methods for *Listeria* detection. Few published studies have addressed these issues. There is conflicting advice from regulatory agencies regarding size of the sampling area and methods for detection (USFDA/CFSAN, 2017; USFDA/CFSAN, 2015; USDA FSIS, 2012; Carpentier & Barre, 2012). Additionally, addressing comparative recovery of swabbing devices from different surface materials has not been well studied. Previous research has shown that environmental swabbing devices (such as a sponge-stick pre-moistened with buffered

peptone water, pre-moistened environmental swabs, and a Copan foam spatula) are capable of detecting *Listeria* spp. on neoprene rubber, high density polyethylene, and stainless steel surfaces at low (100 CFU/250 cm^2) concentrations (Lahou & Uyttendaele, 2014) with the possibility of food residues influencing recovery rates due to enhanced fitness (Kusumaningrum et al., 2002; Takahashi et al., 2011). Nyachuba and Donnelly, (2007) compared the efficacy of three enrichment methods and one enumeration method to detect and isolate *L. monocytogenes* at low (0.1 CFU/cm^2 for inoculum with uninjured cells and 0.1-10 CFU/cm^2 for inoculum with injured cells) levels from dairy environmental surfaces including brick, dairy board, stainless steel, and epoxy resin. These authors found that efficacy of sampling methods and environmental sampling devices depends on the surfaces type, where the modified USDA enrichment method was more efficient in *L. monocytogenes* recovery followed by the selective USDA/FSIS method, then ISO 11290-1, and lastly, the 3M™ Petrifilm™ Environmental *Listeria* Plate method. This study also found variation in recovery by swabbing device, where the environmental sponge was most effective at recovering *L. monocytogenes* from surfaces, followed by the 3M™ Quick Swab, and lastly the M-Vac System. Lahou & Uyttendaele (2014) reported similar results, where recovery of *L. monocytogenes* varied by swab type. *L. monocytogenes* was undetected with the 3M™ Sponge-Stick in 11.1% of samples (n=27), in 7.5% of samples (n=27) with Copan Foam Spatula, and 3.7% of samples (n=27) with the environmental sponge after air drying for 1 hour following inoculation. These studies show that proper selection of testing methods or environmental sampling devices have a significant impact on the recovery of *L. monocytogenes*. Hence, effective

performance of swabbing devices and enrichment methods used to detect *Listeria* spp. on dairy environmental surfaces requires further investigation.

Dairy processors face many choices when selecting testing formats and swab formats to conduct environmental monitoring of *Listeria* spp. in dairy processing facilities. Therefore, this study was conducted to validate the efficacy of three environmental swab formats for the detection of *L. monocytogenes* and *Listeria innocua* (*L. innocua*) on four environmental surfaces (dairy brick, stainless steel, food-grade plastic, and wood) used in dairy processing when using standard cultural methods . The performance of methods and swabs was also tested on samples from naturally contaminated environments to assess performance including inclusivity of recovery of diverse *L. monocytogenes* subtypes. This evaluation will assist dairy processors, particularly artisan cheesemakers, with selection of sensitive and reliable detection procedures.

Methods

Preparation of *Listeria* spp. Strains

Listeria spp. (*L.m.* 19115, *L.m.* 1042B , and *L. innocua*) were selected based upon their source of origin as specified in Table 1 to include a representative population of *Listeria* spp. typically found in dairy processing environments. Strains were prepared as stock cultures by inoculating 1ul of purified culture into10 ml of Trypticase soy broth (TSB) and grown for 24 ± 2 h hours at 35 ± 2°C. Cultures were then mixed into sterile vials as 40% culture and 60% glycerol for preservation and stored at -80°C as previously described (Nyarko et al., 2017).

Preparation of Bacterial Strains

Listeria spp. cold stocks were streaked onto CHROMagar™ (chromogenic *Listeria* base agar (DRG International, Springfield NJ) and incubated for 18-24 h at 35°± 2°C. After adequate growth, one colony was selected from the CHROMagar™ plate and grown in Brain Heart Infusion (BHI) broth and incubated for 18-24 h at 35 ± 2°C. A 1ml aliquot of culture was then added to 99ml of BHI and incubated at 24± 2 h at 35 ± 2°C. Subsequently, high (10^6-10^7 CFU/cm^2) and low (0.01-1 CFU/ cm^2) target inoculum concentrations of *L. innocua 18* Green Fluorescent Protein (GFP), *L.m.* ATTC® 19115 and *L.m.* DUP-1042B strains were enumerated by completing serial dilutions and plating onto 3M™ Aerobic Plate Count (APC) Petrifilm™ (3M™ Microbiology, Saint Paul, MN).

Environmental Materials

This study compared four environmental surfaces (Dairy brick [DB], stainless steel [SS], food-grade high density polypropylene (i.e. plastic) [FGPP], and wood [W]; n=27/surface type at high concentrations; n=405/surface type at low concentrations). Wood samples were prepared from seasoned spruce wooden shelves obtained from a local artisan cheesemaker. Each material was cut into 100 cm^2 sections, thoroughly washed, and sterilized by autoclaving at 121°C for 90 minute and 15-minute cycles prior to use as described by Nyachuba and Donnelly (2007).

Sampling Methods

Three environmental sponge swab formats were evaluated: 1. World Bioproducts EZ Reach™ sponge sampler (World Bioproducts©, Bothell WA) pre-moistened with 10 ml Dey-Engley (D/E) neutralizing broth (WPDE) (Polyurethane) (USFDA/CFSAN, 2017) or 2. HiCap (HC) neutralizing broth (WPHC) (World Bioproducts©, Bothell WA), and 3. 3M™ Sponge-sticks with 10 ml Dey-Engley (D/E) neutralizing broth (3M™ Microbiology, Saint Paul, MN) (Cellulose) as recommended by FDA BAM (U.S. FDA, 2017). The efficacy of recovery of *Listeria* spp. from DB, SS, P, and W surfaces was compared for each sponge swab method by taking a pre-moistened sponge (with 10 ml of D/E or HC) from a sterile bag and hand massaging per manufacturer's instructions prior to swabbing the 100 cm^2 surface using the "meandering movement" (Lahou & Uyttendaele, 2014). The sponge swab was aseptically placed back into the sterile bag and hand massaged for 1 minute prior to further processing. All swab formats were performed on three replicates of each surface per strain and concentration (Nyachuba & Donnelly, 2007).

Recovery and Enumeration of *Listeria* spp. at High Concentrations

Each surface was inoculated with 1 ml of *L. innocua* 18 (GFP) and *L. monocytogenes* ATTC® 19115 and DUP-1042B at an initial target concentration of (10^6-10^7 CFU/ cm^2). Inoculated surfaces were then swabbed (Figure 1) with each of the environmental sponge swabs and enumerated by completing serial dilutions and plating 1 ml of broth onto duplicate 3M™ APC Petrifilm™ (3M™ Microbiology, Saint Paul, MN) that were incubated for 24 ± 2 h at 35 ± 2°C. Red indicator colonies were counted to establish concentrations.

Recovery of *Listeria* spp. at Low Concentrations

The 3M™ Environmental *Listeria* Plating method and the modified FDA (mFDA), FDA (U.S. FDA, 2017) , dual (MOPS-BLEB) enrichment (D'Amico & Donnelly, 2008), and modified USDA (mUSDA) (Nyachuba & Donnelly 2007) enrichment methods were used to compare sensitivity of recovery of *Listeria* spp. between environmental swabs (Figure 1).

The mUSDA and dual MOPS-BLEB dual enrichment methods both require a primary enrichment step using University of Vermont (UVM) broth (Becton, Dickinson and Co., Franklin Lakes, NJ) (USDA/FSIS 2006) and *Listeria* Repair Broth (LRB) (Busch & Donnelly, 1992), and Buffered *Listeria* Enrichment Broth (BLEB) (Neogen Food Safety Lansing, MI) (D'Amico & Donnelly, 2009), respectively. Samples were incubated at 30°± 2°C for 24 ± 2 h (Figure 1). BLEB was used for the primary and only enrichment step for the modified FDA (mFDA) and FDA methods This enrichment broth requires Acriflavin and Nalidixic Acid stock solutions at 0.5% (w/v) and Cycloheximide at a final concentration of 1% (w/v). The mFDA method required the addition of all three antibiotics to BLEB immediately prior to sample enrichment, while the FDA method required the addition of antibiotics after 4 hours of non-selective preincubation to promote repair of injured *Listeria*.

A 50µl aliquot of the primary enrichments were added to Demi Fraser (Becton, Dickinson and Co. Franklin Lakes, NJ) (ISO 11290-1, 1996) and 100 µl aliquot was added to Morpholinepropanesulfonic acid buffered *Listeria*-enrichment broth (MOPS-BLEB) secondary enrichments, respectively and incubated at 35 ± 2°C for 24 ± 2 h.

After enrichment, 100 µl were plated onto Chromogenic *Listeria* selective agar (CHROMagar™, DRG International, Springfield NJ), where a streak for isolation was performed, and plates were incubated for 18-24 h at 35 ± 2°C to confirm presence or absence of growth based upon standard colony morphology (small, metallic, turquoise colonies with halo to detect *L. monocytogenes* and without a halo to detect *L. innocua*).

The performance of 3M™ Petrifilm™ Environmental *Listeria* (EL*)* Plates (adapted from 3M™ Petrifilm™ EL Plate Interpretation Guide 2006) was also evaluated. Buffered Peptone Water (BPW) was added to the sample and left at ambient temperature for 1 hour before 3 ml aliquots were plated onto the EL plates and incubated for 36 ± 2 h at 35 ± 2°C. Enumeration of growth was used to confirm presence or absence of *Listeria* spp.

Electron Microscopy Imaging (MI)

Microscopy Imaging was used to qualitatively compare recovery of *Listeria spp.* from surfaces between environmental swabs. The LeicaMZ16F Stereomicroscope was used to detect the fluorescence of the *L. innocua* 18 GFP inoculum and capture images at 5x and 11.5x magnification. Each surface (DB, P, SS, W) was spot inoculated at high concentrations and an image was taken before and after swabbing.

Farm Environmental Sampling

Environmental sampling a local dairy farm producing milk for artisan cheese manufacture was conducted to verify swab format performance outside of a controlled laboratory setting. Surfaces similar to those tested in the laboratory were targeted to establish efficacy of sponge swabs for the detection of *Listeria* spp. Barn surfaces

included plastic, stainless steel, wood, and concrete [C] (as a replacement for dairy brick). A replicated sampling plan (Figure 2) was used for each swab format and surface. Samples were swabbed onto CHROMagar© *Listeria* in duplicate after they were enriched using dual MOPS-BLEB and mUSDA enrichment methods. Samples were also assayed for *Listeria* identification using the DuPont Qualicon BAX Q7 system (BAX PCR; DuPont Qualicon Wilmington, DE).

Ribotyping

The Dupont Riboprinter Microbial Characterization System (Qualicon Inc.) was used to further explore subtype diversity of recovered *Listeria* spp. as a function of surfaces, swabs, and enrichment/isolation media. The proprietary RiboExplorer software (V.2.0.3121.0) produces Dupont Identifications (DUP-IDS) from fragment patterns of band intensity and position. These DUP-IDS were used to observe ribotype diversity within the dairy farm environment (D'Amico & Donnelly, 2008; Sauders et al., 2006; Sauders et al. 2004; Weidman et al. 1997).

Statistical Analysis

Statistical analyses were completed using the IBM SPSS Statistics program Version 24. Logistic regression and Pearson chi-square cross-tabulation tests were used to determine the statistical significance of interactions between independent variables (surface, swab, method, strain, and concentration) and correlations between results for *Listeria* recovery at low concentrations, respectively. ANOVA tests were completed to establish statistical significance of enumeration results for *Listeria* inoculated to surfaces at at high concentrations between independent variables. Following ANOVA, POST

HOC Bonferonni tests were applied to determine whether or not the difference between means of swab formats or surface types were statistically significant.

Results

Recovery of *Listeria* spp. From Surfaces

This study examined efficacy of *Listeria* recovery and interactive effects from 4 surfaces (W, DB, FGPP and SS), 3 swab formats (3M^{TM}, WBDE, WBHC), 5 detection methods mUSDA, MOPS BLEB, FDA, mFDA and 3M^{TM} ELP), 3 strains (*L. m* 19115; *L.m*. 1042B and *L. innocua*), and 3 concentrations 0.01 CFU/cm^2, 0.1 CFU/cm^2, and 1 CFU/cm^2). When using all surfaces, swab formats, methods, strains, and concentrations combined, a total of 1,620 samples were collected for analysis., where 81.3% (1,317/1,620) of total samples were positive for *Listeria* spp recovery.

When observing total recovery results by concentration at low levels, results by surface and method were statistically significant ($p<0.001$), while results by swab and strain were not (Table 2). When concentrations of 0.01, 0.1, and 1 CFU/cm^2 were applied to material surfaces, *Listeria* spp. were recovered from 52.2% (282/540), 92.6% (500/540) and 99.1% (535/540) of total samples respectively, when using all surfaces, swab formats, methods, and strains. Of these samples, *Listeria* spp. were recovered from: 14.8% (20/135), 77% (104/135), and 96.3% (130/135) of wooden surfaces; 52.3% (71/135) 97.7% (131/135), 100% (135/135) of dairy brick surfaces; 73.3% (99/135), 99.3% (134/135), and 100% (135/135) of plastic surfaces; and 68.1% (92/135), 97% (131/135), and 100% (135/135) of stainless steel surfaces, when applied at initial concentrations of 0.01, 0.1, and 1 CFU/cm^2, respectively. Of the methods, *Listeria* spp.

were recovered from 74.1% (80/108), 93.5% (101/108), and 100% (108/108) of surfaces using the mUSDA enrichment method; 50% (54/108), 96.3% (107/108), and 96.3% (107/108) of surfaces using the dual (MOPS-BLEB) enrichment method; 50% (54/108), 96.3% (107/108), and 96.3% (107/108) of surfaces using the primary FDA enrichment method; 73.1% (78/108), 94.4% (102/108), and 96.3% (107/108) of surfaces using the mFDFA enrichment method; and 14.8% (16/108), 76.8% (83/108), and 98.1% (106/108) of surfaces using the 3M™ Petrifilm™ ELP enumeration method at concentrations of 0.01, 0.1, and 1 CFU/cm^2, respectively. When comparing recovery results by swab, *Listeria* spp. was from 52.2% (94/180), 91.6% (165/180) and 98.8% (178/180) of surfaces when using the 3M™ swab; 59.4% (107/180), 95% (171/180) and 100% (180/180) of surfaces when using the WBDE swab; and 45% (81/180), 91% (164/180), and 98.3% (177/180) of surfaces when using the WBHC swab at concentrations of 0.01, 0.1, and 1 CFU/cm^2, respectively. Lastly, variation in recovery results by strain was observed, where *L. monocytogenes* 19115 was recovered from 56.1% (101/180), 91.6% (165/180), and 99.4% (179/180) of surfaces; *L. monocytogenes* 1042B was recovered from 53.3% (96/180), 95.5% (172/180), and 100% (180/180) of surfaces; and *L. innocua* was recovered from 47.2% (85/180), 90.5% (163/180), and 97.7% (176/180) of surfaces at concentrations of 0.01, 0.1, and 1 CFU/cm^2, respectively.

Listeria spp. were recovered from 90.9% (368/405) , 88.4 (358/405), and 83.2 (337/405) of plastic , stainless steel, and dairy brick surfaces respectively, but only 62.7% (254/405) of wooden surfaces ($p<0.001$) (Table 3). Of the surfaces swabbed, 3M™, WBDE, and WBHC recovered *Listeria* spp. from 80.9% (437/540), 84.8% (458/540), and 78.1% (422/540) of samples, respectively ($p<0.05$). Recovery using 3M™

Petrifilm™ EL Plate enumeration, dual MOPS-BLEB, FDA, mFDA, and mUSDA enrichment methods resulted in *Listeria* spp. detection from 63.3% (205/324), 82.7% (268/324), 82.7% (268/324), 88.6% (287/324), and 89.2% (289/324) of samples, respectively (p value<0.001). Concentration also affected recovery rates, where initial levels of 1 CFU/cm^2, 0.1 CFU/cm^2, and 0.01 CFU/cm^2 were recovered from 52.2% (282/540), 92.6% (500/540), and 99.1% (535/540) of samples, respectively ($p<0.001$). . However, no significant differences were observed in recovery of *Listeria* spp. as a function of strain, where *L. monocytogenes* 1042B, *L. monocytogenes* 19115, and *L. innocua* were recovered from 83% (448/540), 82.4% (445/540), and 78.5% (424/540) of samples, respectively.

At low concentrations, the interaction between surface and method was positively correlated ($p<0.05$), while interactions between (i) surface and swab, (ii) method and swab in reference to each surface, and (iii) surface and concentration (with and without 1 CFU/cm^2 concentration to observe difference in significance as most of these samples at this concentration were positive), and (iv) surface and strain were not (Table 4). Specifically, the number of negative results ($p <0.001$) influenced statistical significance of the surface and method interaction, with wood showing the highest degree of variability.

While pairwise comparisons between swab types (when considering all surfaces and strains) at high concentrations were not significantly different, pairwise comparisons between the swab types and surfaces did have statistically significant differences in *Listeria* spp. recovery. (Table 5). Significant differences between the means of 3M™ (7.633± .109 CFU/100 cm^2) and WBDE (7.811± .109 CFU/100 cm^2) were found

($p<0.05$), while the difference between WBDE (7.811± .109 CFU/100 cm^2) and WBHC (7.745± .109 CFU/100 cm^2), and 3M™ (7.633± .109 CFU/100 cm^2) and WBHC (7.745± .109 CFU/100 cm^2) were not (Table 5). The mean difference in recovery between wood (6.797± .056 CFU/100 cm^2) and plastic (8.108± .056 CFU/100 cm^2), wood (6.797± .056 CFU/100 cm^2) and stainless steel (8.092± .056 CFU/100 cm^2), and wood (6.797± .056 CFU/100 cm^2) and dairy brick (7.922± .056 CFU/100 cm^2) surfaces had the greatest variation in *Listeria* spp. recovery ($p<0.001$) (Table 5). The significance of relative performance between swab and surface demonstrates that the device used to swab a particular surface needs to be chosen based on its efficacy and design.

The difference of means between swab formats for each surface type was also analyzed for statistical significance (Table 6). When surfaces were inoculated at high concentrations, there was a statistically significant difference in recovery from dairy brick ($p<0.001$), where differences between 3M™ (7.755± .083/100 cm^2) and WBDE (8.226± .083 /100 cm^2), and WBDE (8.226± .083 /100 cm^2) and WBHC (7.786± .083 /100 cm^2) were significant. Recovery from plastic surfaces was significant ($p<0.05$) as a result of the mean difference between WBDE (8.335± .094/100 cm^2) and WBHC (7.951± .094/100 cm^2) swabs. Wooden surfaces ($p<0.05$) were also associated with significant mean differences, where comparisons between WBDE (6.444± .135/100 cm^2) and 3M™ (6.672± .135/100 cm^2), and WBHC (7.275± .135/100 cm^2) and 3M™ (6.672± .135/100 cm^2) swabs were significant. Significant differences in recovery from stainless steel were not observed, with no significant difference between means obtained by of 3M™, WBDE, and WBHC swabs.

Our microscopy imaging results also qualitatively demonstrated such variation in inoculum recovery at high concentrations from dairy brick, wood, plastic and stainless steel (Figure 3). Wood and dairy brick surfaces have greater porosity, therefore the inoculum was not as readily available, when visually compared to plastics and stainless steel.

Table 7 summarizes the recovery of *Listeria* spp. from each method at low target concentrations, where recovery is separated by strain (n=108 per strain per method). Both *L. monocytogenes* 19115 and 1042B were recovered from 83.3% (90/108) of samples enriched using the dual (MOPS-BLEB) and primary FDA enrichment method, while *L. innocua* was recovered from 81.5% (88/108) of samples. When comparing the efficacy of the mUSDA, and 3M^{TM} EL Plate methods, *L. monocytogenes* 19115 was recovered from 93.5% (101/108), 90.7% (98/108) and 61.1% (66/108) of samples, *L. monocytogenes* 1042B was recovered from 90.7% (98/108), 90.7% (98/108) and 66.7% (72/108) of samples, and *L. innocua* was recovered from 81.5% (88/108), 86.1% (93/108) and 62.0% (67/108) of samples, respectively. In comparison to other methods, the mFDA method showed the greatest variation of positive recovery results between *Listeria* spp. strains ($p<0.05$).

The recovery of *Listeria* spp. from all surfaces by swab type at low concentrations is summarized in Table 8, where recovery is separated by strain (n=180 per swab type per strain). Comparative results of strains showed that 3M^{TM}, WBDE, and WBHC swab types recovered *L. monocytogenes* 19115 from 83.3% (150/180), 88.3% (159/180), and 75.6% (136/180) of samples; *L .monocytogenes* 1042B from 80.6% (145/180), 83.9%

(151/180), and 84.4% (152/180) of samples, and *L. innocua* from 78.9% (142/180), 82.2% (148/180), and 74.4% (134/180) of samples, respectively. In comparison to other swabs, the WBHC swab showed the greatest variation of positive recovery results between *Listeria* spp. strains ($p<0.05$).

Lastly, Table 9 summarizes *Listeria* spp. recovery by surface at low concentrations, where recovery is separated by strain (n=135 per method per strain). Results show that *Listeria* spp. had the lowest recovery from wood surfaces with recovery rates of 67.4% (91/135), 65.2% (88/135), and 55.6% (75/135) for *L. m.* 19115, *L.m.* 1042B, and *L. innocua,* respectively. Comparative results of strains from DB, FGPP, and SS surfaces showed that *L. monocytogenes* 19115 was recovered from 85.2% (115/135), 88.9% (120/135), and 88.1% (119/135) of surfaces, *L. monocytogenes* 1042B was recovered from 83.7% (113/135), 94.8% (128/135), and 88.1% (119/135) of surfaces, and *L. innocua* from 80.7% (109/135), 88.9% (120/135), and 88.9% (120/135) of surfaces, respectively. No statistically significant differences between recovery of strains were established for any of the surface types.

Farm Environmental Sampling

Farm environmental sampling was performed using MOPS-BLEB and mUSDA enrichment methods. The MOPS-BLEB enrichment method was used because it is the standard culturing method required by Dupont's BAX System, and the mUSDA method was used as it demonstrated superior detection of the five standard enrichment methods used in our laboratory studies. For farm environmental sampling, the experimental design consisted these 2 detection methods, in addition to 4 surfaces (W, DB, FGPP and

SS), and 3 swab formats (3M™, WBDE, WBHC). When using all surfaces, swab formats, and methods combined, a total of 144 samples were collected from dairy farm environments, where 72.9% (105/144) of total samples tested positive for *Listeria* spp. (Table 10). Of these 105 samples that tested positive, *L. monocytogenes* alone, *L. innocua* alone, and *L. monocytogenes* and *L. innocua* together, were recovered from 8.3% (12/144), 35.4% (51/144), and 29.2% (42/144) of samples, respectively, when using all surfaces, swab formats, methods, and strains.

Listeria spp. was recovered from 41.7% (15/36), 94.4% (34/36), 94.5% (34/36), and 61.1% (22/36) of wood, concrete (DB alternative), plastic, and stainless steel surfaces, respectively, where *L. innocua* was recovered more frequently than *L. monocytogenes* ($p<0.001$)(Table 10). Of samples tested, 5.6% (2/36), 16.7% (6/36), and 19.4% (7/36) of wooden surfaces; 13.9% (5/36), 44.4% (16/36), and 36.1% (13/36) of concrete (DB alternative) surfaces; 5.6% (2/36), 55.6%(20/35), and 33.3% (12/35) of plastic surfaces; and 8.3% (3/36), 25% (9/36), and 27.8% (10/36) of stainless steel surfaces showed presence of *L. monocytogenes,, L. innocua*, and both *L. monocytogenes*/*L. innocua*, respectively. No recovery of *Listeria* spp. was observed for 58.3% (21/36), 5.6% (2/36), 5.6% (2/36), and 38.9% (14/36) of wood, concrete (DB alternative), plastic, and stainless steel surfaces, respectively).

Slight differences in recovery by swab format (68.8% for WBHC (33/48), 79.2% (38/48) for WBDE, versus 70.8% (34/48) for 3M™) for all surfaces were also observed (Table 10). Of swabs tested, 3M™ recovered 8.3% (4/48), 33.3% (16/48), and 29.2% (14/48), WBDE recovered 6.3% (3/48), 33.3% (16/48), and 39.6% (19/48), and WBHC recovered 10.4% (5/48), 39.6% (19/48), and 18.8% (9/48) of *L. monocytogenes, L.*

innocua, and *L monocytogenes.* and *L. innocua*, respectively.

The mUSDA method showed slightly higher recovery of *Listeria* spp. (75% (54/72)) from farm environmental surfaces when compared to the dual enrichment method (70.8% (51/72)) (Table 10). Out of the two methods, dual enrichment (MOPS-BLEB) recovered 4.2% (3/72) , 31.9% (23/72), and 34.7% (25/72) and mUSDA recovered 12.5% (9/72), 38.9% (28/72) and 23.6% (17/72) of *L. monocytogenes, L. innocua*, and *L. monocytogenes.* and *L. innocua*, respectively.

Farm environmental sampling result interactions were analyzed by distinguishing *Listeria* spp. presence as *L. monocytogenes, L innocua*, or both (Table 11). Interactions between surface and method, swab and method, or swab and surface were not statistically significant when observing presence of both *Listeria* spp. and *L. innocua*. While surface and method interactions were not significant for the presence of *L. monocytogenes*, swab and surface, and swab and method interactions were ($p \leq 0.05$)

Environmental sampling revealed subtype diversity of *L. monocytogenes* isolates as a function of the swabbing device and detection method, with 10 different subtypes being identified through ribotype analysis: DUP-1039A, DUP 1039E, DUP-1042BA, DUP-1042B, DUP-1045A, DUP-1045B, DUP-1045E, DUP-1047A, DUP-1062B, and DUP-1062C (Table 12). Six of the ten ribotypes (DUP-1042B, DUP-1045B, DUP-1045E, DUP-1045A, DUP-1042A, DUP-1039C) were recovered from plastic surfaces of water troughs; Seven of ten ribotypes (DUP-1042B, DUP-1045B, DUP-1062C, DUP-1039A, DUP-1045A, DUP-1042A, DUP-1039C) were recovered from stainless steel pen fencing; Four of ten ribotypes (DUP-1045B, DUP-1047A, DUP-1062B, DUP-1039A) were recovered from concrete surfaces (farm bed perimeter); and 4 of ten ribotypes

(DUP-1039E, DUP-1045B, DUP-1039E, DUP-1039C) were recovered from wooden wall boards (Table 13). WBDE swabs recovered 8 of ten ribotypes (DUP-1039E, DUP-1042B, DUP-1045B, DUP-1045E, DUP-1039A, DUP-1045A, DUP-1042A, DUP-1039C); 3M™ recovered 7 ribotypes (DUP-1045B, DUP-1062B, DUP-1062C, DUP-1045E, DUP-1039E, DUP-1045A, DUP-1039C); and WBHC recovered 4 ribotypes (DUP-1045B, DUP-1047A, DUP-1039A, DUP-1045A). Comparing selectivity of *L. monocytogenes* ribotypes is useful to inform cheese producers on what methods best reveal the true diversity of *Listeria* subtypes that are present in the dairy farm environment.

Discussion

This comparative evaluation was conducted to explore the relative performance of swab formats and methods for detection of *Listeria* spp. during environmental monitoring. Our data is consistent with other studies showing that the mUSDA method is generally superior regardless of swab type when compared to FDA, mFDA, Dual MOPS-BLEB enrichment, and 3M™ Petrifilm™ ELP enumeration methods (Nyachuba & Donnelly 2007; Pritchard & Donnelly, 1999). Previous research has established that selective agents in enrichment media may mask the detection of cells that have become sublethally injured, therefore using modified enrichment methods could improve the efficacy of recovering injured cells and may explain why the mUSDA method produced more positive results (Bruhn, Vogel, & Gram, 2005; Donnelly 2002). Varied recovery as a result of false negatives could also be from the lack of sensitivity and specificity.

Our work is also consistent with Nyachubua & Donnelly (2007), demonstrating that the 3M™ EL Plate method yielded lower recovery of *Listeria* spp. from surfaces when compared to other standard enrichment methods. The limited performance of this method may be attributed to the use of wooden surfaces, since the 3M™ Petrifilm™ ELP method has only been validated for *Listeria* spp. detection from stainless steel, ceramic tile, and sealed concrete (3M™, 2018). In other studies, this method has proven to be superior or equal to the performance other standard culturing methods in sensitivity and accuracy (Groves and Donnelly, 2005; Horter and Lubrant, 2004). Considering that the 3M™ Petrifilm™ ELP method is more cost effective and is relatively rapid, these findings may encourage cheese makers to increase their sampling size if they use the 3M™ ELP to recover *Listeria* spp. in the processing facility, particularly wooden environmental surfaces.

Ismail et al., (2017) also demonstrated similar trends of *Listeria* recovery from surfaces, reporting that transfer rates of *L. monocytogenes* from perforated plastics (1.09%) and glass (3%) were greater than wooden counterparts. *L. monocytogenes* transfer rates from wooden surfaces to young cheese did not exceed 0.55% (initial concentration of 10^3 and 10^5 CFU/cm^2) due to the porosity of the surface. Lahou & Uyttendaele (2014) had similar findings where there was no significant difference between recovery results of *Listeria* spp. at low concentrations (100 CFU/250 cm^2) from non-porous stainless steel and plastic surfaces.

Clearly, the method used and the surface type and condition of environmental surfaces impacts recovery results (Ismail et al. 2017; Lahou & Uyttendaele 2014; Silva et al. 2008). Understanding the efficacy of the available methods on various surfaces is

beneficial to artisanal cheesemakers to make cost-effective decisions about environmental monitoring resources that best apply to their processing facility and the environmental surfaces that apply to niches within that production environment.

In March of 2014, the FDA implemented new guidelines, stating, "*The use of wooden shelves, rough or otherwise, for cheese ripening does not conform to cGMP requirements, which require that "all plant equipment and utensils shall be so designed and of such material and workmanship as to be adequately cleanable and shall be properly maintained*." (21 CFR 110.40(a)). In response, the artisan cheese communities in the U.S. and the EU contested this guideline and warranted a FDA response three months later in June of 2014, retracting their statement on banning the use of wooden boards for cheese aging. In this statement the FDA specified that their previous mandate on food contact surfaces was not directed towards wooden shelves for cheese aging and did not prohibit their use for artisan cheese production. The FDA clarified its position on the use of wooden boards in cheese aging, writing that "*all plant equipment and utensils shall be so designed and of such material and workmanship as to be adequately cleanable and shall be properly maintained"* (CFR Subsection C. 110.4). Therefore, the inclusion of wooden surfaces in this study for environmental sampling had urgency as a result of the FDA's initial proposed ban targeting wooden shelving for cheese aging.

The artisan cheese industry insures that wooden boards used for cheese aging are cleaned, sanitized, and inspected prior to being used for the next cycle of cheese affinage (Licitra et al., 2014). Any undesired bacteria or yeast that is entrapped in the shelves could lead to a poor-quality cheese product during ripening. Mariani et al., (2007) found that bacteria are capable of penetrating a depth of 1-2cm into the porous matrix of

wooden shelves. Therefore, sanitation protocols should take porosity and bacteria entrapment into consideration and be designed to destroy any bacterium within the wooden board in addition to the topical surface along with verification through environmental monitoring.

While our sampling surface area is consistent with ISO 18593 guidance of at least 100 cm^2, the FDA provides the food industry with a wide range of acceptable guidelines on environmental swabbing methods (Carpentier and Barre, 2012) . The 2015 FDA Testing Methodology for *Listeria* species or *L. monocytogenes* in Environmental Samples has specified that swabbing surfaces in an area of 1 square inch (or 1 ft^2 for sponges per manufacturer's instructions) is sufficient for pathogen testing (USFDA/CFSAN, 2015). The FDA's 2017 Guidance (USFDA/CFSAN, 2017) and the United States Department of Agriculture (USDA) Food Safety Inspection Services (FSIS) *Listeria* Guideline: *Listeria* Control Program: Testing for *L. monocytogenes* or an Indicator Organism (USDA FSIS, 2012) both agree on a sampling surface area size of 1 ft^2. The FDA states that this sampling size is dependent upon the surface that is swabbed and the enrichment methods available as described in 21 CFR 10.117 (FDA/CFSAN, 2017b) On the contrary, the French agency for food environmental and occupational health safety (Anses) and the European Union Reference Laboratory for *Listeria monocytogenes* (EURL *L.m.*) suggests that any given area being sampled should be at least 1,000 cm^2 (Carpentier and Barre, 2012).

In order to control *L. monocytogenes* in processing facilities, cheesemakers need to collect environmental swabs post-cleaning and sanitizing. This will not only validate cleaning methods (Malley et al., 2015; Lahou & Uyttendaele, 2014)., but will also

determine what harborage sites and niches form biofilms when production is occurring and after cleaning and sanitizing (Buchanan et al., 2017). It has been established that *L. monocytogenes* cannot be completely eradicated from processing plants because it is ubiquitous in nature and there are many entry points that can allow the organism into a facility (Buchanan et al., 2017). Therefore, preventing *Listeria* contamination of artisan cheese requires routine and effective environmental monitoring of product contact surfaces within the production environment.

Deciding which environmental swab to use is another important component of an environmental monitoring program, since the swab material and the amount of pressure applied (Lahou & Uyttendaele, 2014; Nyachuba & Donnelly, 2007; Vorst et al., 2004) affects the swabbing devices ability to remove cells from flexible and uneven environmental surfaces that are heavily contaminated (Kusumaningrum et al. 2002). This could result in a lack of sensitivity of standard microbiological analyses by limiting entrapment of bacteria (Moore & Griffith, 2007). Variation in pH, oxygen tension, and nutrient availability could also influence the effectiveness of swabbing devices to recover *Listeria* spp. (Poimenidou et al. 2009). Previous studies have shown that wet surfaces yield a better recovery rate than dry surfaces and may be attributed to inactivated cells when the environment is low in moisture, limiting nutrient availability (Lahou & Uyttendaele 2014; Gomez et al. 2012; Moore et al. 2002). *L. monocytogenes* better attaches to surfaces after drying (especially within the first 20 minutes) (Lahou & Uyttendaele 2014; Beresford et al., 2001) on different environmental materials as indicated by Norwood and Gilmour (2001) suggesting that cellular structures such as flagella, pili, and other extracellular polysaccharides may affect bacteria adhesion and

survival under static conditions (Poimenidou et al. 2009). Hence, it is important for cheesemakers to understand the true diversity of *L. monocytogenes* isolates as a function of swabbing device and detection method since many environmental factors may affect recovery results.

The FDA BAM recommends 3M™ or World Bioproducts© pre-moistened or dry sponge swabs as devices that food producers can use to complete their environmental sampling (USFDA, 2017). The 3M™ Sponge stick uses cellulose material and World Bioproducts uses polyurethane. Polyurethane is known to be stronger and more resistant to tearing, flaking, and fraying. The polyurethane material is also manufactured without toxins, such as quaternary ammonium, which could accrue chemical residue within the sponge and inhibit microbial growth (World Bioproducts, n.d.). Comparably, cellulose is known to be manufactured with those toxic materials, which could lead to chemical residues and subsequently cause false negative results as a result of growth inhibition (Fort, 2011). Cellulose can also break apart and leave small pieces behind when swabbing rough surfaces (Fort, 2011).

Conclusions

This research opens opportunity for further investigation of detection methods and environmental swab formats in addition to the use of sanitizers and drying techniques that may affect recovery of *Listeria* spp. from various surfaces. Discrepancy of results due to the variation in porosity of environmental surfaces and should be taken into consideration by artisan cheesemakers when implementing environmental sampling plans. The concern for cleaning and sanitizing, especially of wooden boards, only emphasizes the need to

establish the efficacy of environmental monitoring devices and methods and apply those findings accordingly to the artisan cheese industry.

Table 1: *Listeria* spp. used to inoculate environmental surfaces

Strain ID	Source	Reference/Source
ATCC 19115 (4b)	Human Subject	(Murray et al., 1926) Pirie
DUP-1042B (4b)	Dairy Farm	CW 193-10 M5-1
Li 18	Food Processing	(Ma, Zhang, & Doyle, 2011) Siliker

Table 2: Summation of results for the recovery of *Listeria* spp. by concentration at low levels

		Target Concentrations of *Listeria* spp. No. Positives/No. Samples Tested (%)			
		0.01 CFU/cm²	**0.1 CFU/cm²**	**1 CFU/cm²**	**Total**
Surface*	W	20/135 (14.8)	104/135 (77)	130/135 (96.3)	254/405 (62.7)
	DB	71/135 (52.3)	131/135 (97.7)	135/135 (100)	337/405 (83.2)
	FGPP	99/135 (73.3)	134/135 (99.3)	135/135 (100)	368/405 (90.9)
	SS	92/135 (68.1)	131/135 (97)	135/135 (100)	358/405 (88.4)
	Total:	282/540 (52.2)	500/540 (92.6)	535/540 (99.1)	1,620/1,620
Swab	3M™	94/180 (52.2)	165/180 (91.6)	178/180 (98.8)	437/540 (80.9)
	WBDE	107/180 (59.4)	171/180 (95)	180/180 (100)	458/540 (84.8)
	WBHC	81/180 (45)	164/180 (91)	177/180 (98.3)	422/540 (78.1)
	Total:	282/540 (52.2)	500/540 (92.6)	535/540 (99.1)	1,620/1,620
Method*	mUSDA	80/108 (74.1)	101/108 (93.5)	108/108 (100)	289/324 (89.2)
	MOPS-BLEB	54/108 (50)	107/108 (96.3)	107/108 (96.3)	268/324 (82.7)
	FDA	54/108 (50)	107/108 (96.3)	107/108 (96.3)	268/324 (82.7)
	mFDA	78/108 (73.1)	102/108 (94.4)	107/108 (96.3)	287/324 (88.6)
	3M™ ELP	16/108 (14.8)	83/108 (76.8)	106/108 (98.1)	205/324 (63.3)
	Total:	282/540 (52.2)	500/540 (92.6)	535/540 (99.1)	1,620/1,620
Strain	*L.m.* 19115	101/180 (56.1)	165/180 (91.6)	179/180 (99.4)	445/540 (82.4)
	L.m. 1042B	96/180 (53.3)	172/180 (95.5)	180/180(100)	448/540 (83)
	L. innocua	85/180 (47.2)	163/180 (90.5)	176/180 (97.7)	424/540 (78.5)
	Total:	282/540 (52.2)	500/540 (92.6)	535/540 (99.1)	1,620/1,620

[a]Chi-square tests were completed on all crosstabulation analyses to determine statistically significant associations (*= $p < 0.05$). DB= dairy brick, FGPP= food grade polypropylene (plastic), SS= stainless steel, W= wood. WBDE=World Bioproducts swab with Dey Engley (DE) or HiCap (HC) neutralizing buffer 3M™ EL Plate= 3M™ Environmental *Listeria* Plates

Table 3: Statistical significance of *Listeria* spp. recovery results by surface, swab type, method, strain, and concentration

Independent Variables		Dependent Variables
		No. Positives/ No. Samples Tested (%)
Surface[a]	DB	337/405 (83.2)**
	FGPP	368/405 (90.9)**
	SS	358/405 (88.4)**
	W	254/405/ (62.7)**
Swab[a]	3M™	437/540 (80.9)*
	WBDE	458/540 (84.8)*
	WBHC	422/540 (78.1)*
Method[a]	3M™ EL Plate	205/324 (63.3)**
	Dual MOPS-BLEB	268/324 (82.7)**
	FDA (Primary)	268/324 (82.7)**
	mFDA	287/324 (88.6)**
	mUSDA	289/324 (89.2)**
Strain[a]	*Lm.* 1042B	448/540 (83)
	L.m. 19115	445/540 (82.4)
	L. innocua	424/540 (78.5)
Concentration[a]	1 CFU/cm^2	535/540 (99.1)**
	0.1 CFU/cm^2l	500/540 (92.6)**
	0.01 CFU/cm^2	282/540 (52.2)**

[a]Chi-square tests were completed on all crosstabulation analyses to determine statistically significant associations (**= $p < 0.001$, *= $p < 0.05$). DB= dairy brick, FGPP= food grade polypropylene (plastic), SS= stainless steel, W= wood. WBDE=World Bioproducts swab with Dey Engley (DE) or HiCap (HC) neutralizing buffer 3M™ EL Plate= 3M™ Environmental *Listeria* Plates

Table 4: Statistical significance of independent variable interactions at low target concentrations

Independent Variables[a]	Sig. (p-value)
Surface and Swab	0.227
Method and Swab	0.584
Surface and Method	0.027*
Surface and Methods negative results[b]	0.000*
Surface and Method positive results[b]	1.000
Swab and Concentration	0.983
Surface and Concentration	0.960
Surface and Concentration (w/o 1 CFU/cm^2)	0.683
Surface and Strain	0.540

[a]Logistic regression tests were completed to determine statistical significance of interactions between independent variables. [b]Pearson chi-square test was completed on crosstabulation analyses to determine statistical significance of associations between independent variables with negative or positive results as a layered variable. DB= dairy brick, FGPP= food grade polypropylene (plastic), SS= stainless steel, W= wood.

Table 5: Statistical significance of enumeration results at high target concentrations between pairwise comparisons of swabs and surfaces		
Independent Variables	**Mean log CFU/100cm^{2a}**	**Pairwise Comparisons**
Swab by (Surface and Strain)		
3M™	7.633± .109	WBDE WBHC
WBDE	7.811± .109	3M™ WBHC
WBHC	7.745± .109	3M™ WBDE
Swab and Surface*		
*Swab**		
3M™	7.633± .049	WBDE* WBHC
WBDE	7.811± .049	3M™* WBHC
WBHC	7.745± .049	3M™ WBDE
*Surface**		
SS	8.092± .056	DB P W*
DB	7.922± .056	P SS W*
P	8.108± .056	DB SS W*
W	6.797± .056	DB* P* SS*

[a]ANOVA tests were completed to determine statistically significant associations between swab, surfaces, and strains; Bonferroni alpha (*$p<0.05$) (adjustment method for pairwise comparisons). DB= dairy brick, FGPP= food grade polypropylene (plastic), SS= stainless steel, W= wood. WBDE/WBHC=World Bioproducts swab with Dey Engley (DE) or HiCap (HC) neutralizing buffer.

Table 6: Statistical significance of enumeration results at high target concentrations between each surface and all swab interactions

Independent Variables	Mean log CFU/100 cm^{2a}	Pairwise Comparisons
Dairy Brick (DB)*		
3M™	7.755± .083	WBDE* WBHC
WBDE	8.226± .083	3M™* WBHC*
WBHC	7.786± .083	3M™* WBDE
Plastic (FGP)*		
3M™	8.038± .094	WBDE WBHC
WBDE	8.335± .094	3M™ WBHC*
WBHC	7.951± .094	3M™ WBDE*
Stainless Steel (SS)		
3M™	8.068± .085	WBDE WBHC
WBDE	8.239± .085	3M™ WBHC
WBHC	7.969± .085	3M™ WBDE
Wood (W)*		
3M™	6.672± .135	WBDE WBHC*
WBDE	6.444± .135	3M™ WBHC*
WBHC	7.275± .135	3M™* WBDE*

[a]ANOVA tests were completed to determine statistically significant associations between swabs and surfaces; Bonferroni alpha (*$p<0.05$) (adjustment method for pairwise comparisons). DB= dairy brick, FGPP= food grade polypropylene (plastic), SS= stainless steel, W= wood. WBDE/WBHC=World Bioproducts swab with Dey Engley (DE) or HiCap (HC) neutralizing buffer.

Table 7: Recovery by method (enriched using mFDA, FDA (BLEB), Dual Enrichment (MOPS-BLEB), or mUSDA or enumerated with 3M™ EL Plates) and strain at low concentrations

		Recovery[a] (No. Positives/No. Samples Tested (%))		
Method	**N[b]**	**19115**	**1042B**	***L. innocua* 18**
FDA (BLEB)	324	90/108 (83.3)	90/108 (83.3)	88/108 (81.5)
DUAL (MOPS-BLEB)	324	90/108 (83.3)	90/108 (83.3)	88/108 (81.5)
mFDA	324	101/108 (93.5)*	98/108 (90.7)*	88/108 (81.5*)
mUSDA	324	98/108 (90.7)	98/108 (90.7)	93/108 (86.1)
3M™ Petrifilm™ ELP	324	66/108 (61.1)	72/108 (66.7)	67/108 (62.0)

*Pearson chi square test determined that recovery by method was statistically significant ($p<0.05$)

[a]Includes % recovery from dairy brick, stainless steel, food grade plastic, and wood

[b]Total number of swab samples taken per strain from surfaces inoculated with 0.01-1 CFU/cm^2 that were enriched using FDA (BLEB), Dual Enrichment (MOPS-BLEB), or mUSDA or enumerated with 3M™ EL Plates

Table 8: Recovery by swab (3M^{tM} environmental swabs, World Bioproducts environmental swabs with Dey Engley neutralizing buffer (WBDE) and HiCap neutralizing buffer (WBHC) and strain at low concentrations

		Recovery[a] (No. Positives/No. Samples Tested (%))		
Swab	**N[b]**	**19115**	**1042B**	***L. innocua* 18**
3M™	540	150/180 (83.3)	145/180 (80.6)	142/180 (78.9)
WB® D/E	540	159/180 (88.3)	151/180 (83.9)	148/180 (82.2)
WB® HC	540	136/180 (75.6)*	152/180 (84.4)*	134/180 (74.4)*

*Pearson chi square test determined that recovery result by method was statistically significant ($p<0.05$)

[a]Includes % recovery from dairy brick, stainless steel, food grade plastic, and wood

[b]Total number of swab samples taken per strain from surfaces inoculated with 0.01-1 CFU/cm^2 CFU/ml that were recovered using 3M™ environmental swabs, World Bioproducts environmental swabs with Dey Engley neutralizing buffer (WBDE) and HiCap neutralizing buffer (WBHC).

Table 9: Recovery by surface (wood (W), dairy brick (DB), food grade polypropylene (FGPP, and stainless steel (SS)) and strain at low concentrations

		Recovery[a] (No. Positives/No. Samples Tested (%))		
Surface	**N[b]**	**19115**	**1042B**	***L. innocua* 18**
W	405	91/135 (67.4)	88/135 (65.2)	75/135 (55.6)
DB	405	115/135 (85.2)	113/135 (83.7)	109/135 (80.7)
FGPP	405	120/135 (88.9)	128/135 (94.8)	120/135 (88.9)
SS	405	119/135 (88.1)	119/135 (88.1)	120/135 (88.9)

*Pearson chi square test determined that recovery by method was statistically significant ($p<0.05$)

[a]Includes % recovery from dairy brick, stainless steel, food grade plastic, and wood

[b]Total number of swab samples taken per strain from surfaces inoculated with 0.01-1 CFU/cm^2 CFU/ml that were enriched using FDA (BLEB), Dual Enrichment (MOPS-BLEB), or mUSDA or enumerated with 3M™ EL Plates

Table 10: Statistical significance of *Listeria* spp. recovery results from farm environmental samples by surface, swab type, and method

Independent Variables		*Dependent Variables*					
			No. Positives/No. Samples Tested				
		N[b]	Negative for *Listeria* spp./ No. Samples Tested	*L.m.*	*L. innocua*	*L.m.* and *Linnocua*	Total *Listeria* spp. Isolated from Samples[b]
Surface[a**]	W	36	21/36 (58.3)	2/36 (5.6)	6/36 (16.7)	7/36 (19.4)	15/36 (41.7)
	C	36	2/36 (5.6)	5/36 (13.9)	16/36 (44.4)	13/36 (36.1)	34/36 (94.4)
	FGPP	36	2/36 (5.6)	2/36 (5.6)	20/36 (55.6)	12/36 (33.3)	34/36 (94.4)
	SS	36	14/36 (38.9)	3/36 (8.3)	9/36 (25)	10/36 (27.8)	22/36 (61.1)
	Total:		39/144 (27.1)	12/144 (8.3)	51/144 (35.4)	42/144 (29.2)	105/144 (72.9)
Swab[a]	3M™	48	14/48 (29.2)	4/48 (8.3)	16/48 (33.3)	14/48 (29.2)	34/48 (70.8)
	WBDE	48	10/48 (20.8)	3/48 (6.3)	16/48 (33.3)	19/48 (39.6)	38/48 (79.2)
	WBHC	48	15/48 (31.3)	5/48 (10.4)	19/48 (39.6)	9/48 (18.8)	33/48 (68.8)
	Total:		39/144 (27.1)	12/144 (8.3)	51/144 (35.4)	42/144 (29.2)	105/144 (72.9)
Method[a]	MOPS-BLEB	72	21/72 (29.2)	3/72 (4.2)	23/72 (31.9)	25/72 (34.7)	51/72 (70.8)
	mUSDA	72	18/72 (25)	9/72 (12.5)	28/72 (38.9)	17/72 (23.6)	54/72 (75)
	Total:		39/144 (27.1)	12/144 (8.3)	51/144 (35.4)	42/144 (29.2)	105/144 (72.9)

[a]Chi-square tests were completed on all crosstabulation analyses to determine statistically significant associations (**= $p < 0.001$, *= $p < 0.05$). [b] Sum of individual samples that tested positive from *L.m.*, *L. innocua*, or *L.m* and *L. innocua*. DB= dairy brick, FGPP= food grade polypropylene (plastic), SS= stainless steel, W= wood. WBDE=World Bioproducts swab with Dey Engley (DE) or HiCap (HC) neutralizing buffer 3M™ EL Plate= 3M™ Environmental *Listeria* Plates

[b]Total number of swab samples taken per strain from surfaces inoculated with 0.01-1 CFU/cm^2 that were enriched using FDA (BLEB), Dual Enrichment (MOPS-BLEB), or mUSDA or enumerated with 3M™ EL Plates.

Table 11: Statistical significance of farm environmental sampling results between independent variable interactions

Independent Variables[a]	Sig. (p-value)		
	***Listeria* spp.**	***L. m.***	***L. innocua***
Surface and Method	0.698	0.667	0.395
Swab and Method	0.868	0.050	0.769
Swab and Surface	0.989	0.018*	0.799

[a]Logistic regression tests were completed to determine statistical significance of interactions between independent variables at low concentrations. *=$p<0.05$

Table 12: *Listeria monocytogenes* Dupont ID Recovered from Surfaces and Swab Formats

DUP ID *L.m.*	Ribotype	Surface				Swab Format		
		Plastic	Stainless Steel	Concrete	Wood	WBDE	WBHC	3M™
1039	1039E				x	x		
1042	1042B		x			x		
1045	1045B	x	x	x	x	x	x	x
1047	1047A			x			x	
1062	1062B			x				x
18595	1062C		x					x
18645	1045E	x				x		x
19157	1039E				x			x
19169	1039A		x	x		x	x	
19178	1045A	x	x			x	x	x
20233	1042A		x			x		
20248	1042B	x	x		x	x		x

x= presence

Table 13: Environmental *Listeria* spp. contamination consistency recovered from surfaces

Surface type	Sample Sites	Isolates Recovered
Plastic	Water Trough	DUP-1042B, DUP-1045B, DUP-1045E, DUP-1045A, DUP-1042A, DUP-1039C
Stainless Steel	Pen Fencing	DUP-1042B, DUP-1045B, DUP-1062C, DUP-1039A, DUP-1045A, DUP-1042A, DUP- 1039C
Concrete	Floor of Pen	DUP-1045B, DUP-1047A, DUP-1062B, DUP-1039A
Wood	Barn Walls	DUP-1039E, DUP-1045B, DUP-1039E, DUP-1039C

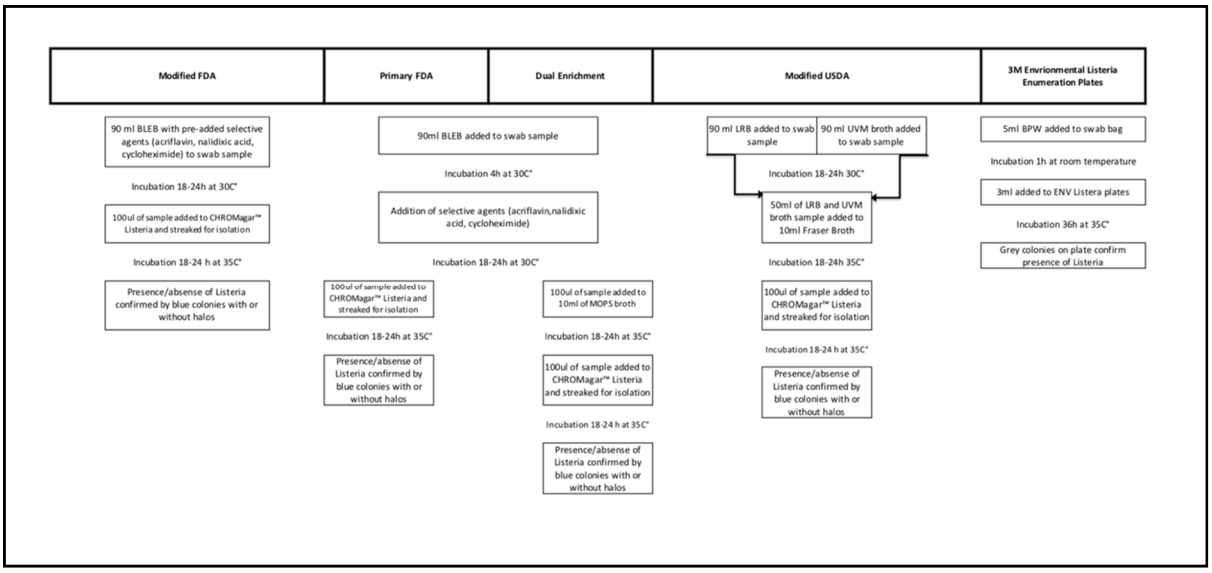

Figure 1: Enrichment Methods against 3MTM Environmental Listeria Enumeration Plates

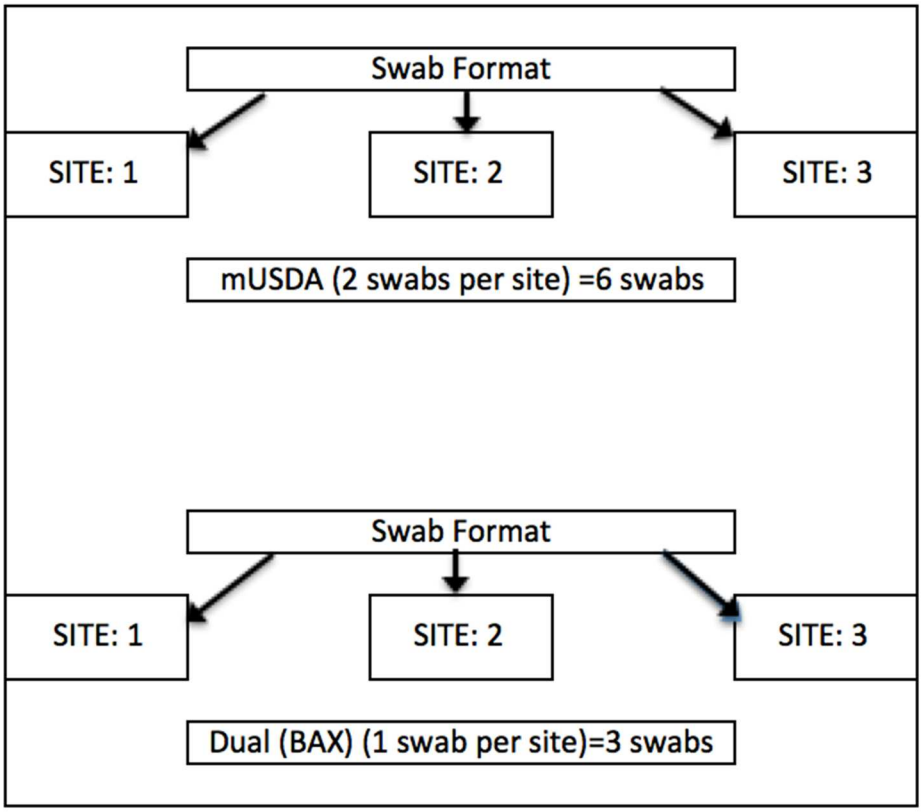

Figure 2: Farm Site-Environmental Sampling Plan

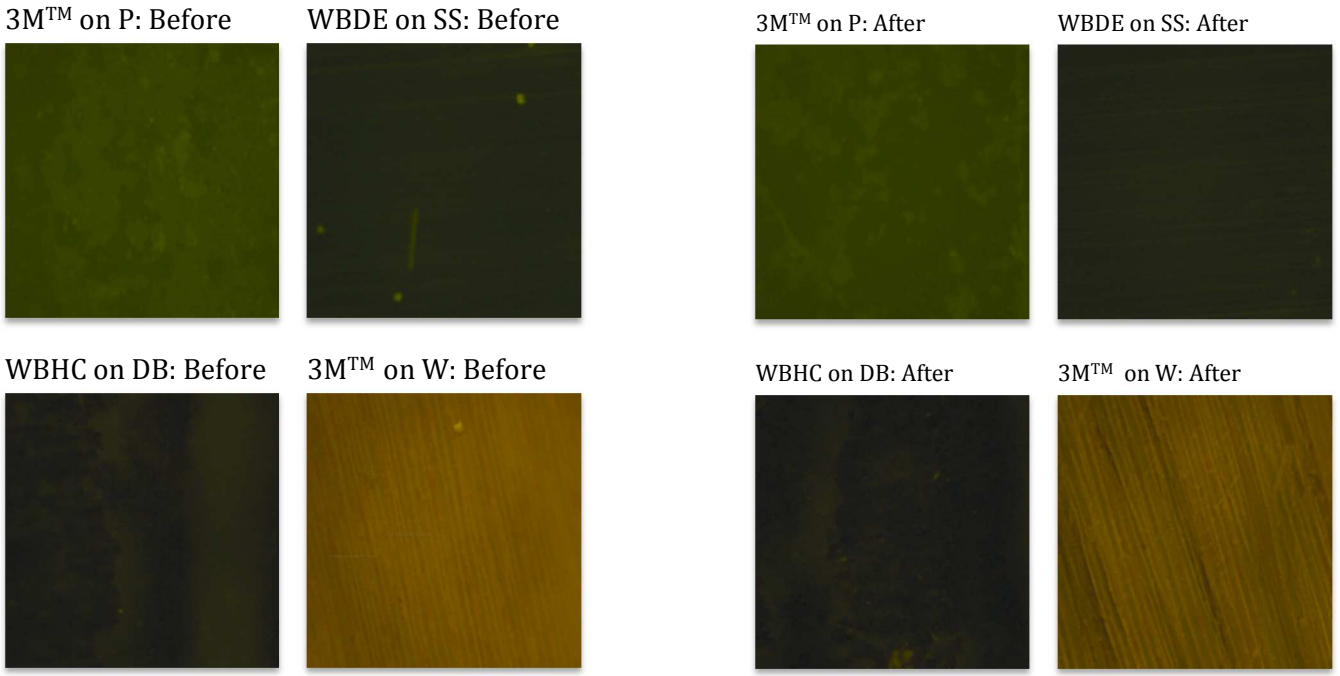

Figure 3: Comparison Using MI Between All Swab Formats at 11.5x Magnification on Surfaces. Left group: before swabbing (top left: 3M™ and P (plastic); top right: WBDE and SS (stainless steel; bottom left: WBHC and DB (diary brick); bottom right: 3M™ and W (wood)). Right group: after swabbing top left: 3M™ from P (plastic); top right: WBDE from SS (stainless steel); bottom left: WBHC from DB (dairy brick); bottom right: 3M™ and W (wood). Other data not shown.

References

3M™. 2018. 3M™ petrifilm™ environmental *Listeria* plates. Available at https://www.3m.com/3M/en_US/company-us/all-3m-products/~/PETRIPA-3M-Petrifilm-Environmental-Listeria-Plates/?N=5002385+3293785686&rt=rud. Accessed on 29 October 2018.

Aviat, F., Gerhards, C., Rodriguez-Jerez, J., Michel, V., Bayon, I. Le, Ismail, R., & Federighi, M. (2016). Microbial safety of wood in contact with food: a review. *Comprehensive reviews in food science and food safety*, *15*(3), 491–505. https://doi.org/10.1111/1541-4337.12199

Beresford, M. R., Andrew, P. W., & Shama, G. (2001). *Listeria monocytogenes* adhers to many materials found in food-processing environments. *Journal of Applied Microbiology*, *90*(6), 1000–1005. https://doi.org/10.1046/j.1365-2672.2001.01330.x

Bruhn, J. B., Vogel, B. F., & Gram, L. (2005). Bias in the *Listeria monocytogenes* enrichment procedure: lineage 2 strains outcompete lineage 1 strains in university of vermont selective enrichments. *Society*, *71*(2), 961–967. https://doi.org/10.1128/AEM.71.2.961

Buchanan, R. L., Gorris, L. G. M., Hayman, M. M., Jackson, T. C., & Whiting, R. C. (2017). A review of *Listeria monocytogenes*: an update on outbreaks, virulence, dose-response, ecology, and risk assessments. *Food Control*, *75*, 1–13. https://doi.org/10.1016/j.foodcont.2016.12.016

Busch, S. V., & Donnelly, C. W. (1992). Development of a repair-enrichment broth for resuscitation of heat-injured *Listeria monocytogenes* and *Listeria innocua*. *Applied and Environmental Microbiology*, *58*(1), 14–20.

Carpentier, B., & Barre, L. (2012). Guidelines on sampling the food processing area and equipment for the detection of *Listeria monocytogenes*. *French Agency for Food, Environmental and Occupational Health Safety*. Available at: https://ec.europa.eu/food/sites/food/files/safety/docs/biosafety_fh_mc_guidelines_on_sampling.pdf. Accessed on 29 October 2018.

Carpentier, B., & Cerf, O. (2011). Review persistence of *Listeria monocytogenes* in food industry equipment and premises. *International J. of Food Microbiology*, *145*(1), 1–8. https://doi.org/10.1016/j.ijfoodmicro.2011.01.005

D'Amico, D. J., & Donnelly, C. W. (2009). Detection, isolation, and incidence of *Listeria* spp. in small-scale artisan cheese processing facilities: a methods comparison. *J. of Food Protection*, *72*(12), 2499–2507.

D'Amico, D. J., & Donnelly, C. W. (2008). Enhanced detection of *Listeria* spp. in farmstead cheese processing environments through dual primary enrichment, PCR,

and molecular subtyping. *J. of Food Protection*, *71*(11), 2239–2248.

D'Amico, D. J., Groves, E., & Donnelly, C. W. (2008). Low incidence of foodborne pathogens of concern in raw milk utilized for farmstead cheese production. *Journal of Food Protection*, *71*(8), 1580-1589.

Donnelly, C.W. (2002). Detection and isolation of *Listeria monocytogenes* from food samples: implications of sublethal injury. *J. AOAC Int*. 85, 495–500

Donnelly, C. W. (2000). Risk Mitigation Through Identification of Source of *L. monocytogenes* in Artisan/Farmstead Cheesemaking Operations, *14*, 1–14. https://doi.org/10.1001/jama.1891.02410890036007

Ferreira V., Wiedmann, M., Teixeira, P., &Stasiewicz, M. J. (2014). *Listeria monocytogenes* persistence in food-associated environments: epidemiology, strain characteristics, and implications for public health. *J. of Food Protection*: January 2014, Vol. 77, No. 1, pp. 150-170.

Fort, R. (2011). White Paper on Cellulose and Polyurethane Sponges for Surface Sampling, (1), 1–5.

Gómez, D., Ariño, A., Carramiñana, J.J., Rota, C., & Yangüela, J. (2012). Comparison of sampling procedures for recovery of *Listeria monocytogenes* from stainless steel food contact surfaces. *J. Food Protect*. 75, 1077–1082. https://doi: 10.4315/0362-028X.JFP-11-421.

Groves, E., & Donnelly, C. W. (2005). Comparison of 3M petrifilm environmental *Listeria* plate vs. standard methods in detecting *Listeria* from environmental surfaces. In *IAFP Annual Meeting, Abstract*.

Harter, E., Wagner, E. M., Zaiser, A., Halecker, S., Wagner, M., & Rychli, K. (2017). Stress survival islet 2, predominantly present in *Listeria monocytogenes* strains of sequence type 121, is involved in the alkaline and oxidative stress responses. *J. of Applied and Environmental Microbiology*, *83*(16), AEM.00827-17. https://doi.org/10.1128/AEM.00827-17

Horter, B., & Lubrant, H. (2004). 3M petrifilm environmental *Listeria* plate for rapid enumeration of *Listeria* from environmental surfaces. In *IAFP Annual Meeting* (p. P138).

Ismail, R., Aviat, F., Gay-Perret, P., Le Bayon, I., Federighi, M., & Michel, V. (2017). An assessment of *L. monocytogenes* transfer from wooden ripening shelves to cheeses: comparison with glass and plastic surfaces. *Food Control*, *73*, 273–280. https://doi.org/10.1016/j.foodcont.2016.08.014

Kovačević, J., Mesak, L. R., &Allen, K. J. (2012). Occurrence and characterization of

Listeria spp. in ready-to-eat retail foods from Vancouver, British Columbia. *J. of Food Microbiology*, *30*(2), 372–378. https://doi.org/10.1016/j.fm.2011.12.015

Kremer, P. H. C., Lees, J. A., Koopmans, M. M., Ferwerda, B., Arends, A. W. M., Feller, M. M., & Bentley, S. D. (2017). Benzalkonium tolerance genes and outcome in *Listeria monocytogenes* meningitis. *Clinical Microbiology and Infection*, *23*(4), 265.e1-265.e7. https://doi.org/10.1016/J.CMI.2016.12.008

Kusumaningrum, H. D., Van Putten, M. M., Rombouts, F. M., & Beumer, R. R. (2002). Effects of antibacterial dishwashing liquid on foodborne pathogens and competitive microorganisms in kitchen sponges. *Journal of Food Protection*, *65*(1), 61–65. https://doi.org/10.4315/0362-028X-65.1.61

Lahou, E., & Uyttendaele, M. (2014). Evaluation of three swabbing devices for detection of *Listeria monocytogenes* on different types of food contact surfaces. *Int. J. Environ*. 11, 804-814. http://doi:10.3390/ijerph110100804

Licitra, G., Lortal, S., & Valence, F. (2014). Wooden tools: reservoirs of microbial biodiversity in traditional cheesemaking. *Microbiology Spectrum*, *2*(1), 1–8. https://doi.org/10.1128/microbiolspec.CM-0008-2012

Ma, L., Zhang, G., & Doyle, M. P. 2011. Green fluorescent protein labeling of *Listeria, Salmonella, and Escherichia coli O157:H7* for safety-related studies. *PLoS ONE*, *6*(4), 1–7. https://doi.org/10.1371/journal.pone.0018083

Malley, T. J. V, Butts, J., & Wiedmann, M. 2015. Seek and destroy process: *Listeria monocytogenes* process controls in the ready-to-eat meat and poultry industry. *Journal of Food Protection*, *78*(2), 436–445. https://doi.org/10.4315/0362-028X.JFP-13-507

Mariani, C., Briandet, R., Chamba, J.-F., Notz, E., Carnet-Pantiez, A., Eyoug, R. N., & Oulahal, N. 2007. Biofilm ecology of wooden shelves used in ripening the french raw milk smear cheese reblochon de savoie. *Journal of Dairy Science*, *90*(4), 1653–1661. https://doi.org/10.3168/JDS.2006-190

Maurel, B., Mastracci, T., Spear, R., Hertault, A., Azzaoui, R., Sobocinski, J., & Haulon, S. 2016. Branched and fenestrated options to treat aortic arch aneurysms. *Journal of Cardiovascular Sugery (Torino), 57*, 686–697. https://doi.org/10.1128/AEM.70.8.4458

Moore, G., & Griffith, C. 2002. A comparison of surface sampling methods for detecting coliforms on food contact surfaces. *Food Microbiol,* 19, 65–73.

Moore, G., & Griffith, C. 2007. Problems associated with traditional hygiene swabbing: the need for in-house standardization. *Journal of Applied Microbiology*, *103*(4),

1090–1103. https://doi.org/10.1111/j.1365-2672.2007.03330.x

Nightingale, K. K., Schukken, Y. H., Nightingale, C. R., Fortes, E. D., Ho, A. J., Her, Z., Grohn Y.T., McDonough P.L.,Wiedmann, M. (2004). Ecology and Transmission of. *Society*, *70*(8), 4458–4467. https://doi.org/10.1128/AEM.70.8.4458

Norwood, D. E., & Gilmour, A. (2001). The differential adherence capabilities of two *Listeria monocytogenes* strains in monoculture and multispecies biofilms as a function of temperature. *Letters in Applied Microbiology*, *33*(4), 320–324. https://doi.org/10.1046/j.1472-765X.2001.01004.x

Nyachuba, D. G., & Donnelly, C. W. (2007). Comparison of 3M™ petrifilm™ environmental *Listeria* plates against standard enrichment methods for the detection of *Listeria monocytogenes* of epidemiological significance from environmental surfaces. *Journal of Food Science*, *72*(9), 346–354. https://doi.org/10.1111/j.1750-3841.2007.00554.x

Nyarko, E., Kniel, K. E., Zhou, B., Millner, P. D., Luo, Y., Handy, E. T., & Sharma, M. (2017). *Listeria monocytogenes* persistence and transfer to cantaloupes in the packing environment is affected by surface type and cleanliness. *Food Control*. https://doi.org/10.1016/j.foodcont.2017.09.033

Pan, Y., Breidt, F., & Kathariou, S. (2006). Resistance of *Listeria monocytogenes* biofilms to sanitizing agents in a simulated food processing environment. *Applied and Environmental Microbiology*, *72*(12), 7711–7717. https://doi.org/10.1128/AEM.01065-06

Poimenidou, S., Belessi, C. A., Giaouris, E. D., Gounadaki, A. S., Nychas, G. E., & Skandamis, P. N. (2009). *Listeria monocytogenes* attachment to and detachment from stainless steel surfaces in a simulated dairy processing environment. *Applied and Environmental Microbiology,75*(22), 7182-7188. doi:10.1128/aem.01359-09

Pritchard, T. J., Beliveau, C. M., Flanders, K. J., & Donnelly, C. W. (1994). Increased incidence of *Listeria* species, *57*(September), 770–775.

Pritchard, T. J., & Donnelly, C. W. (1999). Combined secondary enrichment of primary enrichment broths increases *Listeria* detection. *Journal of Food Protection*, *62*(5), 532–535. https://doi.org/10.4315/0362-028X-62.5.532

Sauders, B. D., K. Mangione, C. Vincent, J. Schermerhorn, C. M. Farchione, N. B. Dumas, D. Bopp, L. Kornstein, E. D. Fortes, K. Windham, & M. Wiedmann. (2004). Distribution of *Listeria monocytogenes* molecular subtypes among human and food isolates from New York State shows persistence of human disease-associated *Listeria monocytogenes* strains in retail environments. *J. Food Prot.* 67:1417–1428.

Sauders, B. D., M. Z. Durak, E. Fortes, K. Windham, Y. Schukken, A. J. Lembo, B. Akey, K. K. Nightingale, & M. Wiedmann. (2006). Molecular characterization of *Listeria monocytogenes* from natural and urban environments. *J. Food Prot.* 69:93–105.

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., & Griffin, P. M. (2011). Foodborne illness acquired in the United States-major pathogens. *Emerging Infectious Diseases*, *17*(1), 7–15. https://doi.org/10.3201/eid1701.P11101

Sheth, I., Li, F., Hur, M., Laasri, A., De Jesus, A. J., Kwon, H. J., & Chen, Y. (2018). Comparison of three enrichment schemes for the detection of low levels of desiccation-stressed *Listeria* spp. from select environmental surfaces. *Food Control*, *84*, 493–498.https://doi.org/10.1016/j.foodcont.2017.08.022

Silva, S., Teixeira, P., Oliveira, R., & Azeredo, J. (2008). Adhesion to and viability of *Listeria monocytogenes* on food contact surfaces. *Journal of Food Protection*, *71*(7), 1379–1385. Available at http://www.ncbi.nlm.nih.gov/pubmed/18680936.Accessed on 29 October 2018.

Takahashi, H., Kuramoto, S., Miya, S., & Kimura, B. (2011). Desiccation survival of *Listeria monocytogenes* and other potential foodborne pathogens on stainless steel surfaces is affected by different food soils. *Food Control*, *22*(3–4), 633–637. Available at https://doi.org/10.1016/j.foodcont.2010.09.003. Accessed 29 October 2018

U.S Center for Disease Control (CDC) (2017). "Multistate Outbreak of Listeriosis Linked to Soft Raw Milk Cheese Made by Vulto Creamery". Available at https://www.cdc.gov/listeria/outbreaks/soft-cheese-03-17/index.html. Accessed on 29 October 2018.

U.S Department of Agriculture/ Food Safety and Inspection Service (FSIS). (2012). Compliance Guideline : Controlling *Listeria monocytogenes* in post-lethality exposed ready-to-eat meat and poultry products, (January), 2–43. Available at http://www.fsis.usda.gov/wps/wcm/connect/d3373299-50e6-47d6-a577-e74a1e549fde/Controlling_LM_RTE_Guideline_0912?MOD=AJPERES. Accessed 29 October 2018.

U.S Food and Drug Administration (FDA). (2017).Bacterialogical Analytical Manual (BAM): Detection and Enumeration of *Listeria monocytogenes* Chapter 10. https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm071400.htm. Accessed on 29 October 2018.

U.S Food and Drug Administration (FDA) Center for Food Safety and Applied Nutrition (USFDA/CFSAN) (2015). Testing Methodology for *Listeria* species or *L. monocytogenes* in Environmental Samples, (October), 1–11.

U.S Food and Driug Administration, Center for Food Safety and Applied Nutrition. (USFDA/CFSAN). (2017). Control of *Listeria monocytogenes* in ready-to-eat foods: guidance for industry draft guidance. *International Journal of Pharmaceutical & Biological Archives*, *3*(Draft Guidance), 1–49. Available at https://www.fda.gov/downloads/food/guidanceregulation/guidancedocumentsregulatoryinformation/ucm535981.pdf. Accessed on 29 October 2018.

U.S Food and Drug Administration (FDA) Center for Food Safety and Applied Nutrition (USFDA/CFSAN). (2018). FSMA final rule for preventive controls for human food. Available at https://www.fda.gov/food/guidanceregulation/fsma/ucm334115.htm. Accessed on 29 October 2018.

U.S. Food and Drug Administration. Department of Health & Human Services (USFDA/HHS) (2017). FDA Form 483 "Vulto creamery inspectional observation report"

U.S Food and Drug Administration. Department of Health and Human Services (USFDA/HHS). (2018). Code of Federal Regulation Title 21. Current Good Manufacturing Practice in Maufacturing, Packing, or Holding Human Food. www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?CFRPart=112. Accessed November 3, 2018.

Vorst, K. L., Todd, E. C. D., & Ryser, E. T. (2004). Improved quantitative recovery of *Listeria monocytogenes* from stainless steel surfaces using a one-ply composite tissue. *Journal of Food Protection*, *67*(10), 2212–2217. Available at http://www.ncbi.nlm.nih.gov/pubmed/15508632. Accessed on 29 October 2018.

Wiedmann, M., Bruce, J. L., Keating, C., & Johnson, A. E., McDonough, P. L., and Batt, C. A. (1997). Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* line-ages with differences in pathogenic potential. Infect. Immun. 65:2707–2716.

World Bioproducts (n.d.). "The Polyurethane Advantage". Available at http://www.worldbioproducts.com/polyurethane.html. Accessed on 20 October 2018.

Comprehensive Bibliography

3M™. 2018. 3M™ petrifilm™ environmental *Listeria* plates. Available at https://www.3m.com/3M/en_US/company-us/all-3m-products/~/PETRIPA-3M-Petrifilm-Environmental-Listeria-Plates/?N=5002385+3293785686&rt=rud. Accessed on 29 October 2018.

Archer, D. L. (2018). The evolution of FDA's policy on *Listeria monocytogenes* in ready-to-eat foods in the United States. *Current Opinion in Food Science*, *20*, 64–68. https://doi.org/https://doi.org/10.1016/j.cofs.2018.03.007

Aarnisalo, K., Autio, T., Sjoberg, A.-M., Lunden, J., Korkeala, H., & Suihko, M.-L. (2003). Typing of *Listeria monocytogenes* isolates originating from the food processing industry with automated ribotyping and pulsed-field gel electrophoresis. *Journal of Food Protection*, *66*(2), 249–255. https://doi.org/10.4315/0362-028X-66.2.249

Abarenkov, K., Henrik Nilsson, R., Larsson, K.-H., Alexander, I. J., Eberhardt, U., Erland, S., … Kõljalg, U. (2010). The UNITE database for molecular identification of fungi – recent updates and future perspectives. *New Phytologist*, *186*(2), 281–285. https://doi.org/10.1111/j.1469-8137.2009.03160.x

Abdul-Mutalib, N.-A., Amin Nordin, S., Osman, M., Ishida, N., Tashiro, K., Sakai, K., … Shirai, Y. (2015). Pyrosequencing analysis of microbial community and food-borne bacteria on restaurant cutting boards collected in Seri Kembangan, Malaysia, and their correlation with grades of food premises. *International Journal of Food Microbiology*, *200*, 57–65. https://doi.org/10.1016/J.IJFOODMICRO.2015.01.022

Abdul-Raouf, U. M., Beuchat, L. R., & Ammar, M. S. (1993). Survival and growth of *Escherichia coli* O157:H7 on salad vegetables. *Applied and Environmental Microbiology*, *59*(7), 1999 LP-2006. Retrieved from http://aem.asm.org/content/59/7/1999.abstract

Agbaje, M., Begum, R. H., Oyekunle, M. A., Ojo, O. E., & Adenubi, O. T. (2011). Evolution of *Salmonella* nomenclature: a critical note. *Folia Microbiologica*, *56*(6), 497–503. https://doi.org/10.1007/s12223-011-0075-4

Ahmad, R., Jilani, G., Arshad, M., Zahir, Z. A., & Khalid, A. (2007). Bio-conversion of organic wastes for their recycling in agriculture: an overview of perspectives and prospects. *Annals of Microbiology*, *57*(4), 471–479. https://doi.org/10.1007/BF03175343

Ahmed, R., Soule, G., Demczuk, W. H., Clark, C., Khakhria, R., Ratnam, S., … Rodgers, F. G. (2000). Epidemiologic Typing of *Salmonella* enterica Serotype Enteritidis in a Canada-Wide Outbreak of Gastroenteritis due to Contaminated Cheese. *Journal of Clinical Microbiology*, *38*(6), 2403–2406. Retrieved from https://jcm.asm.org/content/38/6/2403

Allard, M. W., Strain, E., Melka, D., Bunning, K., Musser, S. M., Brown, E. W., & Timme, R. (2016). Practical Value of Food Pathogen Traceability through Building a Whole-Genome Sequencing Network and Database. *Journal of Clinical Microbiology*, *54*(8), 1975 LP-1983. Retrieved from http://jcm.asm.org/content/54/8/1975.abstract

Allerberger, F., Kreidl, P., Dierich, M. P., Klingsbichel, E., Jenewein, D., Mader, C., … Berghold, C. (2000). *Salmonella* enterica serotype Oranienburg infections associated

with consumption of locally produced Tyrolean cheese. *Euro Surveillance : Bulletin Europeen Sur Les Maladies Transmissibles = European Communicable Disease Bulletin*, *5*(11), 123—126. Retrieved from http://europepmc.org/abstract/MED/12631963

Allison, S. D., & Vitousek, P. M. (1998). Extracellular enzyme activities and carbon chemistry as drivers of tropical plant litter decomposition. *Biotropica*, *36*(3), 285–296. https://doi.org/10.1111/j.1744-7429.2004.tb00321.x

Almeida, G., Magalhães, R., Carneiro, L., Santos, I., Silva, J., Ferreira, V., … Teixeira, P. (2013). Foci of contamination of *Listeria monocytogenes* in different cheese processing plants. *International Journal of Food Microbiology*, *167*(3), 303–309. https://doi.org/10.1016/j.ijfoodmicro.2013.09.006

Altekruse, S. F., Timbo, B. B., Mowbray, J. C., Bean, N. H., & Potter, M. E. (1998). Cheese-associated outbreaks of human illness in the United States, 1973 to 1992: sanitary manufacturing practices protect consumers. *Journal of Food Protection*, *61*(10), 1405–1407. https://doi.org/10.4315/0362-028X-61.10.1405

American Cheese Society (ACS). (2014). Position statement on the safety of aging cheese on wood; issued in response to the recent food and drug administration (FDA) statement on the use of wooden shelves for cheese aging. http://campaign.r20.constantcontact.com/render?ca=7bbd724d-fd30-4d4f-ac09-3817c2f59aa6&c=198439d0-1d97-11e3-9012-d4ae527557ea&ch=19e65700-1d97-11e3-901a-d4ae527557ea. Accessed 8.2.2015.

Anderson, M., Jaykus, L. A., Beaulieu, S., & Dennis, S. (2011). Pathogen-produce pair attribution risk ranking tool to prioritize fresh produce commodity and pathogen combinations for further evaluation (P3ARRT). *Food Control*, *22*(12), 1865–1872. https://doi.org/10.1016/j.foodcont.2011.04.028

Angelo, K. M., Conrad, A. R., Saupe, A., Dragoo, H., West, N., Sorenson, A., … Jackson, B. R. (2017). Multistate outbreak of *Listeria monocytogenes* infections linked to whole apples used in commercially produced, prepackaged caramel apples: United States, 2014–2015. *Epidemiology and Infection*, *145*(5), 848–856. https://doi.org/DOI: 10.1017/S0950268816003083

Applications, W. (2002). Dairy Pipeline, *14*(2), 1–12.

Arimi, S. M., Ryser, E. T., Pritchard, T. J., & Donnelly, C. W. (1997). Diversity of *Listeria* ribotypes recovered from dairy cattle, silage, and dairy processing environments. *Journal of Food Protection*, *60*(7), 811–816. https://doi.org/10.4315/0362-028X-60.7.811

Arul, L., Benita, G., & Balasubramanian, P. (2008). Functional insight for beta-glucuronidase in *Escherichia coli* and Staphylococcus sp. RLH1. *Bioinformation*, *2*(8), 339–343. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2478733&tool=pmcentrez&rendertype=abstract

Aruscavage, D., Phelan, P. L., Lee, K., & LeJeune, J. T. (2010). Impact of changes in sugar exudate created by biological damage to tomato plants on the persistence of *Escherichia coli* O157:H7. *Journal of Food Science*, *75*(4). https://doi.org/10.1111/j.1750-3841.2010.01593.x

Autio, T., Hielm, S., Miettinen, M., Sjoberg, A., Aarnisalo, K., & Bjorkroth, J. et al. (1999). Sources of *Listeria monocytogenes* con- tamination in a cold-smoked

rainbow trout processing plant detected by pulsed-Weld gel electrophoresis typing. *Applied and Environmental Microbiology*, *65*(1), 150–155.

Aviat, F., Gerhards, C., Rodriguez-Jerez, J., Michel, V., Bayon, I. Le, Ismail, R., & Federighi, M. (2016). Microbial safety of wood in contact with food: a review. *Comprehensive reviews in food science and food safety*, *15*(3), 491–505. https://doi.org/10.1111/1541-4337.12199

Bachmann, H. P., & Spahr, U. (1995). The Fate of Potentially Pathogenic Bacteria in Swiss Hard and Semihard Cheeses Made from Raw Milk. *Journal of Dairy Science*, *78*(3), 476–483. https://doi.org/10.3168/jds.S0022-0302(95)76657-7

Bae, D., Seo, K. S., Zhang, T., & Wang, C. (2013). Characterization of a potential *Listeria monocytogenes* virulence factor associated with attachment to fresh produce. *Applied and Environmental Microbiology*, *79*(22), 6855–6861. https://doi.org/10.1128/AEM.01006-13

Baker, C. A., Rubinelli, P. M., Park, S. H., Carbonero, F., & Ricke, S. C. (2016). Shiga toxin-producing *Escherichia coli* in food: Incidence, ecology, and detection strategies. *Food Control*, *59*, 407–419. https://doi.org/10.1016/j.foodcont.2015.06.011

Barnard, R. L., Osborne, C. A., & Firestone, M. K. (2013). Responses of soil bacterial and fungal communities to extreme desiccation and rewetting. *ISME Journal*, *7*(11), 2229–2241. http://doi.org/10.1038/ismej.2013.104

Bellio, A., Bianchi, D. M., Vitale, N., Vernetti, L., Gallina, S., & Decastelli, L. (2018). Behavior of *Escherichia coli* O157:H7 during the manufacture and ripening of Fontina Protected Designation of Origin cheese. *Journal of Dairy Science*, *101*(6), 4962–4970. https://doi.org/10.3168/jds.2017-13458

Beno, S. M., Stasiewicz, M. J., Andrus, A. D., Ralyea, R. D., Kent, D. J., Martin, N. H., … Boor, K. J. (2016). Development And Validation Of Pathogen Environmental Monitoring Programs For Small Cheese Processing Facilities. *Journal of Food Protection*, *79*(12), 2095–2106. https://doi.org/10.4315/0362-028X.JFP-16-241

Beresford, M. R., Andrew, P. W., & Shama, G. (2001). *Listeria monocytogenes* adhers to many materials found in food-processing environments. *Journal of Applied Microbiology*, *90*(6), 1000–1005. https://doi.org/10.1046/j.1365-2672.2001.01330.x

Besser, R., Lett, S., Weber, J., Doyle, M., & Barrett, T. (1993). An outbreak of diarrhea and hemolytic uremic syndrome from *Escherichia coli* O157: H7 in fresh-pressed apple cider. *Jama*, *30333*, 7–10. Retrieved from http://jama.jamanetwork.com/article.aspx?articleid=405799

Beuchat, L. R., & Brackett, R. E. (1991). Behavior of *Listeria monocytogenes* inoculated into raw tomatoes and processed tomato products. *Applied and Environmental Microbiology*, *57*(5), 1367–1371.

Beuchat, L. R., Farber, J. M., Garrett, E. H., Harris, L. J., Parish, M. E., Suslow, T. V, & Busta, F. F. (2001). Standardization of a Method To Determine the Ef cacy of Sanitizers in Inactivating Human Pathogenic Microorganisms on Raw Fruits and Vegetables. *Journal of Food Protection*, *64*(7), 1079–1084. https://doi.org/10.1111/j.1541-4337.2003.tb00034.x

Beumer, R. R., Te Giffel, M. C., Anthonie, S., & Cox, L. J. (2009). *Listeria monocytogenes*. *Foodborne Pathogens: Hazards, Risk Analysis and Control: Second Edition*. https://doi.org/10.1533/9781845696337.2.675

Beumer, R. , Giffel, M. , Kok, M. and Rombouts, F. (1996), Confirmation and identification of *Listeria* spp. Letters in Applied Microbiology, 22: 448-452. doi:10.1111/j.1472-765X.1996.tb01200.x

Beuvier, E., & Duboz, G. (2013). The Microbiology of Traditional Hard and Semihard Cooked Mountain Cheeses. *Microbiology Spectrum*, *1*(1), CM-0006-2012. https://doi.org/10.1128/microbiolspec.CM-0006-2012.f1

Bezanson, G. S., Khakhria, R., Duck, D., & Lior, H. (1985). Molecular analysis confirms food source and simultaneous involvement of two distinct but related subgroups of *Salmonella* typhimurium bacteriophage type 10 in major interprovincial *Salmonella* outbreak. *Applied and Environmental Microbiology*, *50*(5), 1279–1284. Retrieved from https://aem.asm.org/content/50/5/1279

Bone, F. J., Bogie, D., & Morgan-Jones, S. C. (1989). Staphylococcal food poisoning from sheep milk cheese. *Epidemiology and Infection*, *103*(3), 449–458. https://doi.org/10.1017/S0950268800030855

Bonsaglia, E. C. R., Silva, N. C. C., Fernades Júnior, A., Araújo Júnior, J. P., Tsunemi, M. H., & Rall, V. L. M. (2014). Production of biofilm by *Listeria monocytogenes* in different materials and temperatures. *Food Control*, *35*(1), 386–391. https://doi.org/10.1016/j.foodcont.2013.07.023

Borucki, M. K., Peppin, J. D., White, D., Loge, F., & Call, D. R. (2003). Variation in biofilm formation among strains of *Listeria monocytogenes*. *Applied and Environmental Microbiology*, *69*(12), 7336–7342. https://doi.org/10.1128/AEM.69.12.7336

Borucki, M. K., Reynolds, J., Gay, C. C., McElwain, K. L., Kim, S. H., Knowles, D. P., & Hu, J. (2004). Dairy farm reservoir of *Listeria monocytogenes* sporadic and epidemic strains. *Journal of Food Protection*, *67*(11), 2496–2499. https://doi.org/10.4315/0362-028X-67.11.2496

Brandl, M. T., & Amundson, R. (2008). Leaf age as a risk factor in contamination of lettuce with *Escherichia coli* O157:H7 and *Salmonella* enterica. *Applied and Environmental Microbiology*, *74*(8), 2298–2306. https://doi.org/10.1128/AEM.02459-07

Bray, J., & Curtis, J. (1957). An Ordination of the Upland Forest Communities of Southern Wisconsin. *Ecological Monographs*, *27*(4), 325–349. https://doi.org/10.2307/1942268

Brochier, V., Gourland, P., Kallassy, M., Poitrenaud, M., & Houot, S. (2012). Occurrence of pathogens in soils and plants in a long-term field study regularly amended with different composts and manure. *Agriculture, Ecosystems and Environment*, *160*, 91–98. https://doi.org/10.1016/j.agee.2011.05.021

Brooks, J. C., Martinez, B., Stratton, J., Bianchini, A., Krokstrom, R., & Hutkins, R. (2012). Survey of raw milk cheeses for microbiological quality and prevalence of foodborne pathogens. *Food Microbiology*, *31*(2), 154–158. https://doi.org/10.1016/j.fm.2012.03.013

Brosch, R., Chen, J., & Luchansky, J. B. (1994). Pulsed-field fingerprinting of listeriae: Identification of Genomic divisions for *Listeria monocytogenes* and their correlation with serovar. *Applied and Environmental Microbiology*, *60*(7), 2584–2592.

Bruhn, J. B., Vogel, B. F., & Gram, L. (2005). Bias in the *Listeria monocytogenes* Enrichment Procedure: Lineage 2 Strains Outcompete Lineage 1 Strains in

University of Vermont Selective Enrichments. *Society*, *71*(2), 961–967. https://doi.org/10.1128/AEM.71.2.961

Bryan, F. L. (1983). Epidemiology of Milk-Borne Diseases. *Journal of Food Protection*, *46*(7), 637–649. https://doi.org/10.4315/0362-028X-46.7.637

Buazzi, M. M., Johnson, M. E., & Marth, E. H. (1992). Survival of *Listeria monocytogenes* During the Manufacture and Ripening of Swiss Cheese. *Journal of Dairy Science*, *75*(2), 380–386. https://doi.org/10.3168/jds.S0022-0302(92)77772-8

Buchanan, R. L., Gorris, L. G. M., Hayman, M. M., Jackson, T. C., & Whiting, R. C. (2017). A review of *Listeria monocytogenes*: An update on outbreaks, virulence, dose-response, ecology, and risk assessments. *Food Control*, *75*, 1–13. https://doi.org/10.1016/j.foodcont.2016.12.016

Burall, L. S., Grim, C. J., & Datta, A. R. (2017). A clade of *Listeria monocytogenes* serotype 4b variant strains linked to recent listeriosis outbreaks associated with produce from a defined geographic region in the US. *PloS One*, *12*(5), e0176912–e0176912. https://doi.org/10.1371/journal.pone.0176912

Busch, S. V., & Donnelly, C. W. (1992). Development of a repair-enrichment broth for resuscitation of heat-injured *Listeria monocytogenes* and *Listeria* innocua. *Applied and Environmental Microbiology*, *58*(1), 14–20.

Caldwell, B. A. (2005). Enzyme activities as a component of soil biodiversity: A review. *Pedobiologia*, *49*(6), 637–644. https://doi.org/10.1016/j.pedobi.2005.06.003

Callon, C., Berdagué, J. L., Dufour, E., & Montel, M. C. (2005). The Effect of Raw Milk Microbial Flora on the Sensory Characteristics of Salers-Type Cheeses. *Journal of Dairy Science*, *88*(11), 3840–3850. https://doi.org/10.3168/jds.S0022-0302(05)73069-1

Campbell, A., & Gibbard, J. (1944). The survival of E. typhosa in cheddar cheese manufactured from infected raw milk. *Canadian Journal of Public Health*, *35*(4), 158–164.

Canillac, N., & Mourey, A. (2001). Antibacterial activity of the essential oil of Picea excelsa on Listeria, *Staphylococcus aureus* and coliform bacteria. *Food Microbiology*, *18*(3), 261–268. https://doi.org/10.1006/fmic.2000.0397

Carey, C. M., Kostrzynska, M., & Thompson, S. (2009). *Escherichia coli* O157:H7 stress and virulence gene expression on Romaine lettuce using comparative real-time PCR. *Journal of Microbiological Methods*, *77*(2), 235–242. https://doi.org/10.1016/j.mimet.2009.02.010

Carpentier, B., & Barre, L. (2012). Guidelines on sampling the food processing area and equipment for the detection of *Listeria monocytogenes*. *French Agency for Food, Environmental and Occupational Health Safety*. Available at: https://ec.europa.eu/food/sites/food/files/safety/docs/biosafety_fh_mc_guidelines_on_sampling.pdf. Accessed on 29 October 2018.

Carpentier, B., & Cerf, O. (2011). Review - Persistence of *Listeria monocytogenes* in food industry equipment and premises. *International Journal of Food Microbiology*, *145*(1), 1–8. https://doi.org/10.1016/j.ijfoodmicro.2011.01.005

Carter, J. M., Lin, A., Clotilde, L., & Lesho, M. (2016). Rapid, multiplexed characterization of Shiga Toxin-Producing *Escherichia coli* (STEC) isolates using suspension array technology. *Frontiers in Microbiology*, *7*(MAY), 1–6.

https://doi.org/10.3389/fmicb.2016.00439
Center for Disease Control and Prevention (CDC). (2017a). Listeriosis: People at Risk. https://www.cdc.gov/listeria/risk.html. Accessed November 4, 2018.
Center for Disease Control and Prevention(CDC) (2017b). "Multistate Outbreak of Listeriosis Linked to Soft Raw Milk Cheese Made by Vulto Creamery". Available at https://www.cdc.gov/listeria/outbreaks/soft-cheese-03-17/index.html. Accessed on 29 October 2018.
Center for Disease Control and Prevention (CDC). (2016).)Multistate Outbreak of Listeriosis Linked to Blue Bell Creameries Products (Final Update) https://www.cdc.gov/listeria/outbreaks/ice-cream-03-15/index.html. Accessed November 4, 2018.
Center for Disease Control and Prevention (CDC). (2015). Multistate Outbreak of Listeriosis Linked to Soft Cheeses Distributed by Karoun Dairies, Inc. (Final Update). https://www.cdc.gov/listeria/outbreaks/soft-cheeses-09-15/index.html. Accessed November 4, 2018.
Centers for Disease Control and Prevention (CDC). (2013). National Antimicrobial Resistance Monitoring System: Enteric Bacteria. Human Isolates Final Report. Available at: https://www.cdc.gov/narms/pdf/2013-annual-report-narms-508c.pdf. Accessed December 3, 2018.
Center for Disesease Control and Prevention (CDC). (2012). Foodborne Diseases Active Surveillance Network. Food Net 2012 Surveillance Report. https://www.cdc.gov/foodnet/PDFs/2012_annual_report_508c.pdf. Accessed December 3, 2018.
Center for Disease Control and Prevention (CDC). (2011). Multistate Outbreak of Listeriosis Associated with Jensen Farms Cantaloupe --- United States, August--September 2011https://www.cdc.gov/mmwr/preview/mmwrhtml/mm6039a5.htm. Accessed November 4, 2018.
Center for Disease Control and Prevention (CDC) (2000). Morbidity and Mortality Report. https://www.cdc.gov/mmwr/PDF/wk/mm4940.pdf. Accessed November 4, 2018.
Cebrián, G., Condón, S., & Mañas, P. (2017). Physiology of the Inactivation of Vegetative Bacteria by Thermal Treatments: Mode of Action, Influence of Environmental Factors and Inactivation Kinetics. *Foods*, *6*(12), 107. https://doi.org/10.3390/foods6120107
Çekiç, S. K., De, J., Jubair, M., & Schneider, K. R. (2017). Persistence of Indigenous *Escherichia coli* in Raw Bovine Manure-Amended Soil. *Journal of Food Protection*, *80*(9), 1562–1573. https://doi.org/10.4315/0362-028X.JFP-17-033
Center for Food Safety and Applied Nutrition. (2017). Control of *Listeria monocytogenes* in Ready-To-Eat Foods: Guidance for Industry Draft Guidance. *International Journal of Pharmaceutical & Biological Archives*, *3*(Draft Guidance), 1–49. Retrieved from https://www.fda.gov/downloads/food/guidanceregulation/guidancedocumentsregulatoryinformation/ucm535981.pdf
Chacha, M., Bojase-Moleta, G., & Majinda, R. R. T. (2005). Antimicrobial and radical scavenging flavonoids from the stem wood of Erythrina latissima. *Phytochemistry*, *66*(1), 99–104. https://doi.org/10.1016/j.phytochem.2004.10.013

Chan, M. M. Y. (2002). Antimicrobial effect of resveratrol on dermatophytes and bacterial pathogens of the skin. *Biochemical Pharmacology*, *63*(2), 99–104. https://doi.org/10.1016/S0006-2952(01)00886-3

Charlton, B. R., Kinde, H., & Jensen, L. H. (1990). Environmental survey for *Listeria* species in California milk processing plants. *Journal of Food Protection*, *53*(3), 198–201. Retrieved from http://www.cabdirect.org/abstracts/19900439663.html;jsessionid=6F67B1F6E6B48710F8A71A7CC9612A5C

Chasseignaux, E., Toquin, M. T., Ragimbeau, C., Salvat, G., Colin, P., & Ermel, G. (2001). Molecular epidemiology of *Listeria monocytogenes* isolates collected from the environment, raw meat and raw products in two poultry- and pork-processing plants. *Journal of Applied Microbiology*, *91*(5), 888–899. https://doi.org/10.1046/j.1365-2672.2001.01445.x

Chen, Y., Burall, L. S., Luo, Y., Timme, R., Melka, D., Muruvanda, T., … Brown, E. W. (2016). *Listeria monocytogenes* in Stone Fruits Linked to a Multistate Outbreak: Enumeration of Cells and Whole-Genome Sequencing. *Applied and Environmental Microbiology*, *82*(24), 7030 LP-7040. Retrieved from http://aem.asm.org/content/82/24/7030.abstract

Chen, Y., & Knabel, S. J. (2007). Multiplex PCR for simultaneous detection of bacteria of the genus Listeria, *Listeria monocytogenes*, and major serotypes and epidemic clones of *L. monocytogenes*. *Applied and Environmental Microbiology*, *73*(19), 6299–6304. https://doi.org/10.1128/AEM.00961-07

Chen, Y., Zhang, W., & Knabel, S. J. (2007). Multi-Virulence-Locus Sequence Typing Identifies Single Nucleotide Polymorphisms Which Differentiate Epidemic Clones and Outbreak Strains of *Listeria monocytogenes* *Journal of Clinical Microbiology*, *45*(3), 835 LP-846. Retrieved from http://jcm.asm.org/content/45/3/835.abstract

Cheng, Y., Siletzky, R. M., & Kathariou, S. (2008). Genomic divisions/lineages, epidemic clones, and population structure. *Handbook of Listeria monocytogenes. CRC Press, Boca Raton, FL*, 337–358.

Cobbold, R. N., Davis, M. A., Rice, D. H., Szymanski, M., Tarr, P. I., Besser, T. E., & Hancock, D. D. (2008). Associations between Bovine, Human, and Raw Milk, and Beef Isolates of Non-O157 Shiga Toxigenic *Escherichia coli* within a Restricted Geographic Area of the United States. *Journal of Food Protection*, *71*(5), 1023–1027. https://doi.org/10.4315/0362-028X-71.5.1023

Cody, S., Abbott, S., Marfin, A., & Et, A. (1999). Two outbreaks of multidrug-resistant *Salmonella* serotype typhimurium dt104 infections linked to raw-milk cheese in northern california. *JAMA*, *281*(19), 1805–1810. Retrieved from http://dx.doi.org/10.1001/jama.281.19.1805

Communicable Disease Surveillance Centre (CDSC). 1999a. *Escherichia coli* O157 associated with eating unpasteurised cheese. *Commun. Dis. Rep. CDR Weekly* 9(13):116.

Confederation, S. (2014). Opinion on the use of wooden shelves for cheese ripening, *41*(0).

Confederation Internationale des Fromagers Detaillants (CIFD). (2014). Note de Synthèse Problématique du critère *E. coli* sur les fromages au lait cru imposé par la

FDA (English translation). http://iccheesemongers.com/wp-content/uploads/synth%c3%a8se-english-translation-vweb.pdf. Accessed 4.17.17.
Correll, W.A. (2014). Letter to American Cheese Society (ACS) with an Update on What Has Been Done with Respect to Non-Toxigenic *Escherichia coli* (*E. coli*) in Raw Milk Cheese, Public Health Service, Food and Drug Administration, College Park, MD.
Cooley, M. B., Chao, D., & Mandrell, R. E. (2006). *Escherichia coli* O157:H7 survival and growth on lettuce is altered by the presence of epiphytic bacteria. *J Food Prot*, *69*(10), 2329–2335. https://doi.org/10.4315/0362-028X-69.10.2329
Cooley, M., Carychao, D., Crawford-Miksza, L., Jay, M. T., Myers, C., Rose, C., Keys C., Farrar J., Mandrell, R. E. (2007). Incidence and tracking of *Escherichia coli* O157:H7 in a major produce production region in California. *PLoS ONE*, *2*(11). http://doi.org/10.1371/journal.pone.0001159
Cruciata, M., Gaglio, R., Scatassa, M. L., Sala, G., Cardamone, C., Palmeri, M., … Settanni, L. (2018). Formation and characterization of early bacterial biofilms on different wood typologies applied in dairy production. *Applied and Environmental Microbiology*, *84*(4), 1–13. https://doi.org/10.1128/AEM.02107-17
Curiale, M. S., & Lewus, C. (1994). Detection of *Listeria monocytogenes* in Samples Containing *Listeria* innocua, *57*, 1048–1051.
Cutler, A.J., Neher, D.A., Weicht, T.R., Sharma, M., and Millner, P. (2018) *Escherichia coli* survival and microbial community dynamics in compost-amended soils. *Microbial Ecology* MECO-D-18-00672 (submitted 10/23/18).
D'Amico, D.J. (2008). Incidence, Ecology, and Fate of Target Foodborne Pathogens in the Cheesemaking Continuum, (October), 1–235. Thesis.
D'Amico, D.J., and Donnelly C.W. (2011). FDA's Domestic and Imported Cheese Compliance Program Results: January 1, 2004- December 31, 2006. *J Food Prot* Trends. 31(4), 216–226.
D'Amico, D. J., & Donnelly, C. W. (2010). Microbiological quality of raw milk used for small-scale artisan cheese production in Vermont: Effect of farm characteristics and practices. *Journal of Dairy Science*, *93*(1), 134–147. https://doi.org/10.3168/jds.2009-2426
D'Amico, D. J., & Donnelly, C. W. (2009). Detection, isolation, and incidence of *Listeria* spp. in small-scale artisan cheese processing facilities: a methods comparison. *Journal of Food Protection*, *72*(12), 2499–2507.
D'Amico, D. J., & Donnelly, C. W. (2008). Enhanced detection of *Listeria* spp. in farmstead cheese processing environments through dual primary enrichment, PCR, and molecular subtyping. *Journal of Food Protection*, *71*(11), 2239–2248.
D'Amico, D. J., Druart, M. J., & Donnelly, C. W. (2014). Comparing the Behavior of Multidrug-Resistant and Pansusceptible *Salmonella* during the Production and Aging of a Gouda Cheese Manufactured from Raw Milk, *77*(6), 903–913. https://doi.org/10.4315/0362-028X.JFP-13-515
D'Amico, D. J., Druart, M. J., & Donnelly, C. W. (2010). Behavior of *Escherichia coli* O157:H7 during the manufacture and aging of Gouda and stirred-curd Cheddar cheeses manufactured from raw milk. *Journal of Food Protection*, *73*(12), 2217–2224.
D'Amico, D. J., Druart, M. J., & Donnelly, C. W. (2008). 60-day aging requirement does

not ensure safety of surface-mold-ripened soft cheeses manufactured from raw or pasteurized milk when *Listeria monocytogenes* is introduced as a postprocessing contaminant. *Journal of Food Protection*, *71*(8), 1563–1571.

D'aoust, J.-Y., Warburton, D. W., & Sewell, A. M. (1985). *Salmonella* typhimurium Phage-Type 10 from Cheddar Cheese Implicated in a Major Canadian Foodborne Outbreak. *Journal of Food Protection*, *48*(12), 1062–1066. https://doi.org/10.4315/0362-028X-48.12.1062

Dalton, C. B., Austin, C. C., Sobel, J., Hayes, P. S., Bibb, W. F., Graves, L. M., … Griffin, P. M. (1997). An Outbreak of Gastroenteritis and Fever Due to *Listeria monocytogenes* in Milk. *New England Journal of Medicine*, *336*(2), 100–106. https://doi.org/10.1056/NEJM199701093360204

Dawson, P., Han, I., Cox, M., Black, C., & Simmons, L. (2007). Residence time and food contact time effects on transfer of *Salmonella* Typhimurium from tile, wood and carpet: Testing the five-second rule. *Journal of Applied Microbiology*, *102*(4), 945–953. https://doi.org/10.1111/j.1365-2672.2006.03171.x

De Buyser, M. L., Dufour, B., Maire, M., & Lafarge, V. (2001). Implication of milk and milk products in food-borne diseases in France and in different industrialised countries. *International Journal of Food Microbiology*, *67*(1–2), 1–17. https://doi.org/10.1016/S0168-1605(01)00443-3

De Cesare, A., Mioni, R., & Manfreda, G. (2007). Prevalence of *Listeria monocytogenes* in fresh and fermented Italian sausages and ribotyping of contaminating strains. *International Journal of Food Microbiology*, *120*(1–2), 124–130. https://doi.org/10.1016/j.ijfoodmicro.2007.06.009

De Jesus, A. J., & Whiting, R. C. (2003). Thermal inactivation, growth, and survival studies of *Listeria monocytogenes* strains belonging to three distinct genotypic lineages. *Journal of Food Protection*, *66*(9), 1611–1617. https://doi.org/10.4315/0362-028X-66.9.1611

DeLaune PB, Moore PA, Daniel TC, Lemunyon JL (2004) Effect of chemical and microbial amendments on ammonia voltalization from composting poultry litter. J Environ Qual 33:728-734.

de las Heras, A., Cain, R. J., Bielecka, M. K., & Vázquez-Boland, J. A. (2011). Regulation of *Listeria* virulence: PrfA master and commander. *Current Opinion in Microbiology*, *14*(2), 118–127. https://doi.org/https://doi.org/10.1016/j.mib.2011.01.005

Department of Health and Human Services. U.S Food and Drug Administration (FDA). (2015). Compliance Program for Domestic and Imported Cheese and Cheese Products. Chapter 3: Foodborne Biological Hazards. (Update of 1998 Food Compliance Program for Domestic and Imported Cheese and Cheese Products. Chapter 3: Foodborne Biological Hazards.). http://www.fda.gov/downloads/Food/ComplianceEnforcement/FoodCompliancePrograms/UCM456592.pdf. Accessed 11.5.2015.

Department of Health and Human Services. U.S. Food and Drug Administration (FDA). (2006). Code of Federal Regulations, chap. 1, subchapter B, title 21, part 133. Cheeses and related cheese products. Section 133.182: soft ripened cheeses. Available at: http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr133.182.

Accessed 9.22.17.
Department of Health and Human Services. U.S. Food and Drug Administration (FDA). (2002). Compliance Policy Guide: ‘‘Filth from Insects, Rodents, and Other Pests in Food’’; Availability. Federal Register, Vol. 67, No. 231 / Monday, December 2, 2002 / Notices. https://www.gpo.gov/fdsys/pkg/FR-2002-12-02/pdf/02-30403.pdf
Department of Health and Human Services. U.S Food and Drug Administration (FDA). (1998). Compliance Program for Domestic and Imported Cheese and Cheese Products. Chapter 3: Foodborne Biological Hazards. http://www.fda.gov/Food/GuidanceComplianceRegulatoryInformation/ComplianceEnforcement/UCM071510.htm. Accessed 8.20.15.
De Reu, K., Grijspeerdt, K., & Herman, L. (2007). A Belgian Survey Of Hygiene Indicator Bacteria And Pathogenic Bacteria In Raw Milk And Direct Marketing Of Raw Milk Farm Products. *Journal of Food Safety*, *24*(1), 17–36. https://doi.org/10.1111/j.1745-4565.2004.tb00373.x
De Valk, H., Delarocque-Astagneau, E., Colomb, G., Ple, S., Godard, E., Vaillant, V., … Desenclos, J.-C. (2000). A community-wide outbreak of *Salmonella* enterica serotype Typhimurium infection associated with eating a raw milk soft cheese in France. *Epidemiology and Infection*, *124*(1), 1–7. https://doi.org/DOI: undefined
Delbes, C., Alomar, J., Chougui, N., Martin, J.-F., & Montel, M.-C. (2006). *Staphylococcus aureus* Growth and Enterotoxin Production during the Manufacture of Uncooked, Semihard Cheese from Cows’ Raw Milk. *Journal of Food Protection*, *69*(9), 2161–2167. https://doi.org/10.4315/0362-028X-69.9.2161
Delmotte, N., Knief, C., Chaffron, S., Innerebner, G., Roschitzki, B., Schlapbach, R., … Vorholt, J. A. (2009). Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *Proceedings of the National Academy of Sciences*, *106*(38), 16428–16433. https://doi.org/10.1073/pnas.0905240106
Dervisoglu, M., & Yazici, F. (2001). Ripening changes of Kulek cheese in wooden and plastic containers. *Journal of Food Engineering*, *48*(3), 243–249. https://doi.org/10.1016/S0260-8774(00)00164-3
Desenclos, J.-C., Bouvet, P., Benz-Lemoine, E., Grimont, F., Desqueyroux, H., Rebiere, I., & Grimont, P. A. (1996). Large outbreak of *Salmonella* enterica serotype paratyphi B infection caused by a goats{\textquoteright} milk cheese, France, 1993: a case finding and epidemiological study. *BMJ*, *312*(7023), 91–94. https://doi.org/10.1136/bmj.312.7023.91
Desmasures, N., & Gueguen, M. (1997). Monitoring the microbiology of high quality milk by monthly sampling over 2 years. *Journal of Dairy Research*, *64*(2), 271–280. https://doi.org/DOI: undefined
Di Grigoli, A., Francesca, N., Gaglio, R., Guarrasi, V., Moschetti, M., Scatassa, M. L., … Bonanno, A. (2015). The influence of the wooden equipment employed for cheese manufacture on the characteristics of a traditional stretched cheese during ripening. *Food Microbiology*, *46*, 81–91. https://doi.org/10.1016/j.fm.2014.07.008
Dieuleveux, V., & Guéguen, M. (1998). Antimicrobial Effects of d-3-Phenyllactic Acid on *Listeria monocytogenes* in TSB-YE Medium, Milk, and Cheese. *Journal of Food Protection*, *61*(10), 1281–1285. https://doi.org/10.4315/0362-028X-61.10.1281
Dineen, S. S., Takeuchi, K., Soudah, J. E., & Boor, K. J. (1998). Persistence of *Escherichia coli* O157:H7 in dairy fermentation systems. *Journal of Food*

Protection®, *61*(12), 1602–1608. https://doi.org/10.4315/0362-028X-61.12.1602
Djordjevic, D. (2002). Microtiter plate assay for assessment of *Listeria monocytogenes* biofilm formation. *Applied and Environmental Microbiology*, *68*(6), 2950–2958. https://doi.org/10.1128/AEM.68.6.2950
Donnelly, C.W. (2002). Detection and isolation of *Listeria monocytogenes* from food samples: implications of sublethal injury. *J. AOAC Int*. 85, 495–500
Donnelly, C. W. (2000). Risk Mitigation Through Identification of Source of *L. monocytogenes* in Artisan/Farmstead Cheesemaking Operations, *14*, 1–14. https://doi.org/10.1001/jama.1891.02410890036007
Donnelly, C. W. (2001). *Listeria monocytogenes*: a Continuing Challenge. *Nutrition Reviews*, *59*(6), 183–194. Retrieved from http://dx.doi.org/10.1111/j.1753-4887.2001.tb07011.x
Donnelly, C. W. (2013). From Pasteur to Probiotics : A Historical Overview of Cheese and Microbes. *Microbiology Spectrum*, *1*(1), CM-0001-2012. https://doi.org/10.1128/microbiolspec.CM-0001-2012.f1
Doyle, M. P. (1991). *Escherichia coli* O157:H7 and its significance in foods. *International Journal of Food Microbiology*, *12*(4), 289–301. https://doi.org/10.1016/0168-1605(91)90143-D
Dreux, N., Albagnac, C., Carlin, F., Morris, C. E., & Nguyen-The, C. (2007). Fate of *Listeria* spp. on parsley leaves grown in laboratory and field cultures. *Journal of Applied Microbiology*, *103*(5), 1821–1827. https://doi.org/10.1111/j.1365-2672.2007.03419.x
Dreux, N., Albagnac, C., Federighi, M., Carlin, F., Morris, C. E., & Nguyen-The, C. (2007). Viable but non-culturable *Listeria monocytogenes* on parsley leaves and absence of recovery to a culturable state. *Journal of Applied Microbiology*, *103*(4), 1272–1281. https://doi.org/10.1111/j.1365-2672.2007.03351.x
Edberg, S. C., E. W. Rice, R. J. Karlin, and M. J. Allen. (2000). *Escherichia coli*: The best biological drinking water indicator for public health protection. *Symp. Ser. Soc. Appl. Microbiol*. (29):106S–116S.
Edgar, R. C. (2013). UPARSE: Highly accurate OTU sequences from microbial amplicon reads. *Nature Methods*, *10*(10), 996–998. https://doi.org/10.1038/nmeth.2604
El-Gazzar, F. E., & Marth, E. H. (1992). *Salmonella*e, Salmonellosis, and Dairy Foods: A Review. *Journal of Dairy Science*, *75*(9), 2327–2343. https://doi.org/10.3168/jds.S0022-0302(92)77993-4
Ellis, A., Preston, M., Borczyk, A., Miller, B., Stone, P., Hatton, B., … Hockin, J. (1998). A community outbreak of *Salmonella* berta associated with a soft cheese product. *Epidemiology and Infection*, *120*(1), 29–35. https://doi.org/DOI: undefined
Emerson, J. B., Keady, P. B., Brewer, T. E., Clements, N., Morgan, E. E., Awerbuch, J., Miller, S.L., and Fierer, N. (2015). Impacts of flood damage on airborne bacteria and fungi in homes after the 2013 Colorado front range flood. *Environmental Science and Technology*, *49*(5), 2675–2684. http://doi.org/10.1021/es503845j
Erickson, M. C., & Doyle, M. P. (2007). Food as a vehicle for transmission of Shiga toxin-producing *Escherichia coli*. *Journal of Food Protection*, *70*(10), 2426–2449. https://doi.org/10.4315/0362-028X-70.10.2426
Erickson, M. C., Webb, C. C., Diaz-Perez, J. C., Phatak, S. C., Silvoy, J. J., Davey, L., … Doyle, M. P. (2010). Surface And Internalized *Escherichia coli* O157:H7 On Field-

Grown Spinach And Lettuce Treated with Spray-Contaminated Irrigation Water. *Journal of Food Protection*, *73*(6), 1023–1029. https://doi.org/10.4315/0362-028X-73.6.1023

European Commission. (2015). Food Law General Principles. http://ec.europa.eu/food/safety/general_food_law/principles/index_en.htm. Accessed 8.10.2015.

European Union (EU). (2005). Commission regulation (EC No 2073/2005 of 15 November 2005 on microbial criteria for foodstuffs. Official Journal of the European Union. L338, 1-26. http://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32005R2073&from=en. Accessed 4.13.17

EU Commission. (2004). Regulation (EC) No 852/2004. *Official Journal of the European Communities*, *2002*(May 2002), 1–54. Retrieved from http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:139:0001:0054:en:PDF

European Food Safety Authority. (2009). Technical specifications for the monitoring and reporting of verotoxigenic *Escherichia coli* (VTEC) on animals and food (VTEC surveys on animals and food) on request of EFSA. *EFSA Journal 2009; 7(11):1366*, *7*(11), 1–43. https://doi.org/10.2903/j.efsa.2009.1366.

European Food Safety Authority. (2013). Scientific Opinion on VTEC-seropathotype and scientific criteria regarding pathogenicity assessment. *EFSA Journal*, *11*(4), 3138. https://doi.org/10.2903/j.efsa.2013.3138

European Food Safety Authority (2016). The European Union summary report on trends and sources of zoonoses, zoonotic agents and food□borne outbreaks in 2015. *EFSA Journal*, *14*(12). https://doi.org/10.2903/j.efsa.2016.4634

European Food Safety Authority. (2007). Monitoring of verotoxigenic *Escherichia coli* (VTEC) and identification of human pathogenic VTEC types Scientific Opinion of the Panel on Biological Hazards. *October*, *579*, 1–61.

Evans J.W., (1968). The General Agreement on Tariffs and Trade. International Organization, 22(01), 72.

Evenson, M. L., Ward Hinds, M., Bernstein, R. S., & Bergdoll, M. S. (1988). Estimation of human dose of staphylococcal enterotoxin A from a large outbreak of staphylococcal food poisoning involving chocolate milk. *International Journal of Food Microbiology*, *7*(4), 311–316. https://doi.org/10.1016/0168-1605(88)90057-8

Ewald, S., & Notermans, S. (1988). Effect of water activity on growth and enterotoxin D production of *Staphylococcus aureus*, *6*, 25–30.

Fagerlund, A. (2017). crossm Cleaning and Disinfection of Biofilms Composed of *Listeria monocytogenes*, *83*(17), 1–21.

FAO. (1996). Corporate Document Depository. http://www.fao.org/docrep/meeting/005/w2198e/w2198e0a.htm. Accessed 8.2.2015

Farber, J. M., & Peterkin, P. I. (1991). *Listeria monocytogenes*, a food-borne pathogen. *Microbiological Reviews*, *55*(3), 476 LP-511. Retrieved from http://mmbr.asm.org/content/55/3/476.abstract

Farber, J. M., & Peterkin, P. I. (2018). *Listeria monocytogenes*: A Food-Borne Pathogen. *Foodborne Diseases*, *55*(3), 157–192. https://doi.org/10.1016/B978-0-12-811444-5.00006-3

Farrokh, C., Jordan, K., Auvray, F., Glass, K., Oppegaard, H., Raynaud, S., … Cerf, O. (2013). Review of Shiga-toxin-producing *Escherichia coli* (STEC) and their

significance in dairy production. *International Journal of Food Microbiology*, *162*(2), 190–212. https://doi.org/10.1016/j.ijfoodmicro.2012.08.008
Feng ', P., & Lampel ', K. A. (2016). Genetic analysis of uidA expression in enterohaemorrhagic Escherichia colr' serotype 01 57 : H7. *Microbiology*, *140*(1), 1–2. https://doi.org/10.1099/13500872-140-8-2101
Ferreira, V., Wiedmann, M., Teixeira, P., & Stasiewicz, M. J. (2014). *Listeria monocytogenes* Persistence in Food-Associated Environments: Epidemiology, Strain Characteristics, and Implications for Public Health. *Journal of Food Protection*, *77*(1), 150–170. https://doi.org/10.4315/0362-028X.JFP-13-150
Flanders, K. J., Pritchard, T. J., & W, C. (1995). Enhanced recovery of *Listeria* from dairy-plant processing environments through combined use of repair enrichment and selective enrichment/detection procedures. *Journal of Food Protection*, *58*(4), 404–409. https://doi.org/10.4315/0362-028X-58.4.404
Fleming, D. W., Cochi, S. L., MacDonald, K. L., Brondum, J., Hayes, P. S., Plikaytis, B. D., … Reingold, A. L. (1985). Pasteurized Milk as a Vehicle of Infection in an Outbreak of Listeriosis. *New England Journal of Medicine*, *312*(7), 404–407. https://doi.org/10.1056/NEJM198502143120704
Flessa, S., Lusk, D. M., & Harris, L. J. (2005). Survival of *Listeria monocytogenes* on fresh and frozen strawberries. *International Journal of Food Microbiology*, *101*(3), 255–262. https://doi.org/10.1016/j.ijfoodmicro.2004.11.010
Florence, A., Christian, G., José□juan, R., Valérie, M., Le, B. I., Rached, I., & Michel, F. (2016). Microbial Safety of Wood in Contact with Food: A Review. *Comprehensive Reviews in Food Science and Food Safety*, *15*(3), 491–505. https://doi.org/doi:10.1111/1541-4337.12199
Fontaine, R. E., Cohen, M. L., Martin, W. T., & Vernon, T. M. (1980). Epidemic Salmonellosis From Cheddar Cheese: Surveillance And Prevention. *American Journal Of Epidemiology*, *111*(2), 247–253. Retrieved from http://dx.doi.org/10.1093/oxfordjournals.aje.a112892
Food Standards Agency and Food Standards Scotland. (2016). UK Working Policy On Detection Of Stec In Food By Official Controls, 1–8.
Fort, R. (2011). White Paper On Cellulose And Polyurethane Sponges For Surface Sampling, (1), 1–5.
Foschino, R., Invernizzi, A., Barucco, R., & Stradiotto, K. (2002). Microbial composition, including the incidence of pathogens, of goat milk from the Bergamo region of Italy during a lactation year. *Journal of Dairy Research*, *69*(2), 213–225. https://doi.org/DOI: 10.1017/S0022029902005459
Fox, E., Hunt, K., O'Brien, M., & Jordan, K. (2011). *Listeria monocytogenes* in Irish Farmhouse cheese processing environments. *International Journal of Food Microbiology*, *145*(SUPPL. 1), S39–S45. https://doi.org/10.1016/j.ijfoodmicro.2010.10.012
Franz, E., Semenov, A. V., Termorshuizen, A. J., De Vos, O. J., Bokhorst, J. G., & Van Bruggen, A. H. C. (2008). Manure-amended soil characteristics affecting the survival of *E. coli* O157:H7 in 36 Dutch soils. *Environmental Microbiology*, *10*(2), 313–327. https://doi.org/10.1111/j.1462-2920.2007.01453.x
Fremaux, B., Delignette-Muller, M. L., Prigent-Combaret, C., Gleizal, A., & Vernozy-Rozand, C. (2007). Growth and survival of non-O157:H7 Shiga-toxin-producing

Escherichia coli in cow manure. *Journal of Applied Microbiology*, *102*(1), 89–99. https://doi.org/10.1111/j.1365-2672.2006.03059.x

Fremaux, B., Prigent-Combaret, C., Delignette-Muller, M. L., Mallen, B., Dothal, M., Gleizal, A., & Vernozy-Rozand, C. (2008). Persistence of Shiga toxin-producing *Escherichia coli* O26 in various manure-amended soil types. *Journal of Applied Microbiology*, *104*(1), 296–304. https://doi.org/10.1111/j.1365-2672.2007.03532.x

Fremaux, B., Prigent-Combaret, C., & Vernozy-Rozand, C. (2008). Long-term survival of Shiga toxin-producing *Escherichia coli* in cattle effluents and environment: An updated review. *Veterinary Microbiology*, *132*(1–2), 1–18. https://doi.org/10.1016/j.vetmic.2008.05.015

Fremaux, B., Raynaud, S., Beutin, L., & Rozand, C. V. (2006). Dissemination and persistence of Shiga toxin-producing *Escherichia coli* (STEC) strains on French dairy farms. *Veterinary Microbiology*, *117*(2–4), 180–191. https://doi.org/10.1016/j.vetmic.2006.04.030

Froman, M. (2014). Office of the United States Trade Representative. 2014 Report on Sanitary and Phytosanitary Measures. (Revised from 1994), 1-116. https://ustr.gov/sites/default/files/FINAL-2014-SPS-Report-Compiled.pdf. Accessed 4.16.17.

FSA. (2001). Food Law, (March), 1–158. Retrieved from https://www.food.gov.uk/sites/default/files/food_law_code_of_practice_2017.pdf

Gaglio, R., Cruciata, M., Di Gerlando, R., Scatassa, M. L., Cardamone, C., Mancuso, I., … Settanni, L. (2016). Microbial activation of wooden vats used for traditional cheese production and evolution of neoformed biofilms. *Applied and Environmental Microbiology*, *82*(2), 585–595. https://doi.org/10.1128/AEM.02868-15

Galinari, É., da Nóbrega, J. E., de Andrade, N. J., & Ferreira, C. L. de L. F. (2014). Microbiological aspects of the biofilm on wooden utensils used to make a Brazilian artisanal cheese. *Brazilian Journal of Microbiology*, *45*(2), 713–720. https://doi.org/10.1590/S1517-83822014000200047

Gasanov, U., Hughes, D., & Hansbro, P. M. (2005). Methods for the isolation and identification of *Listeria* spp. and *Listeria monocytogenes*: A review. *FEMS Microbiology Reviews*, *29*(5), 851–875. https://doi.org/10.1016/j.femsre.2004.12.002

Gay, M., Cerf, O., & Davey, K. R. (1996). Significance of pre□incubation temperature and inoculum concentration on subsequent growth of *Listeria monocytogenes* at 14°C. *Journal of Applied Microbiology*, *81*(4), 433–438. https://doi.org/10.1111/j.1365-2672.1996.tb03530.x

Genigeorgis, C. a. (1989). Present state of knowledge on staphylococcal intoxication. *International Journal of Food Microbiology*, *9*(4), 327–360. https://doi.org/10.1016/0168-1605(89)90100-1

Glass, K. A., Prasad, B. B., Schlyter, J. H., Uljas, H. E., Farkye, N. Y., & Luchansky, J. B. (1995). Effects of Acid Type and Alta^{TM}2341 on *Listeria monocytogenes* in a Queso Blanco Type of Cheese. *Journal of Food Protection*, *58*(7), 737–741. https://doi.org/10.4315/0362-028X-58.7.737

Goepfert, J. M., Olson, N. F., & Marth, E. H. (1968). Behavior of *Salmonella* typhimurium During Manufacture and Curing of Cheddar Cheese. *Applied Microbiology*, *16*(6), 862 LP-866. Retrieved from

http://aem.asm.org/content/16/6/862.abstract
Gómez, D., Ariño, A., Carramiñana, J.J., Rota, C., & Yangüela, J. (2012). Comparison of sampling procedures for recovery of *Listeria monocytogenes* from stainless steel food contact surfaces. *J. Food Protect*. 75, 1077–1082. https://doi: 10.4315/0362-028X.JFP-11-421.
Gómez-Lucía, E., Goyache, J., Orden, J. A., Domenech, A., Javier Hernandez, F., Ruiz-Santa Quiteria, J. A., … Suárez, G. (1992). Growth of *Staphylococcus aureus* and Synthesis of Enterotoxin During Ripening of Experimental Manchego-Type Cheese. *Journal of Dairy Science*, *75*(1), 19–26. https://doi.org/10.3168/jds.S0022-0302(92)77733-9
Gottlieb, S., Wu, V.-C., Wang, Y.-T., Wang, C.-Y., Tsai, I.-J., Wu, K.-D., … Hsueh, P.-R. (2006). Multistate Outbreak of Listeriosis Linked to Turkey Deli Meat and Subsequent Changes in US Regulatory Policy. *Clinical Infectious Diseases*, *42*(1), 66–72. https://doi.org/10.1086/498509
Gough, N. L., & Dodd, C. E. R. (1998). The survival and disinfection of *Salmonella* typhimurium on chopping board surfaces of wood and plastic. *Food Control*, *9*(6), 363–368. https://doi.org/10.1016/S0956-7135(98)00127-3
Gould, L. H., Mungai, E., & Barton Behravesh, C. (2014). Outbreaks Attributed to Cheese: Differences Between Outbreaks Caused by Unpasteurized and Pasteurized Dairy Products, United States, 1998–2011. *Foodborne Pathogens and Disease*, *11*(7), 545–551. https://doi.org/10.1089/fpd.2013.1650
Grace, J. B., Anderson, T. M., Olff, H., & Scheiner, S. M. (2010). On the specification of structural equation models for ecological systems. *Ecological Monographs*, *80*(1), 67–87. http://doi.org/10.1890/09-0464.1
Graves, L. M., & Swaminathan, B. (2001). PulseNet standardized protocol for subtyping *Listeria monocytogenes* by macrorestriction and pulsed-field gel electrophoresis. *International Journal of Food Microbiology*, *65*(1–2), 55–62. https://doi.org/10.1016/S0168-1605(00)00501-8
Graves, L. M., Swaminathan, B., Reeves, M. W., Hunter, S. B., Weaver, R. E., Plikaytis, B. D., & Schuchat, A. (1994). Comparison of ribotyping and multilocus enzyme electrophoresis for subtyping of *Listeria monocytogenes* isolates. *Journal of Clinical Microbiology*, *32*(12), 2936–2943.
Gray, M. J., Gray, M. J., Boor, K. J., & Boor, K. J. (2006). How the Bacterial Pathogen. *Society*, *74*(5), 2505–2512. https://doi.org/10.1128/IAI.74.5.2505
Gray, M. J., Zadoks, R. N., Fortes, E. D., Dogan, B., Cai, S., Chen, Y., … Wiedmann, M. (2004). *Listeria monocytogenes* isolates from foods and humans form distinct but overlapping populations. *Applied and Environmental Microbiology*, *70*(10), 5833–5841. https://doi.org/10.1128/AEM.70.10.5833-5841.2004
Greenwood, M. H., Roberts, D., & Burden, P. (1991). The occurrence of *Listeria* species in milk and dairy products: a national survey in England and Wales. *International Journal of Food Microbiology*, *12*(2), 197–206. https://doi.org/https://doi.org/10.1016/0168-1605(91)90070-6
Gronewold, A. D., & Wolpert, R. L. (2008). Modeling the relationship between most probable number (MPN) and colony-forming unit (CFU) estimates of fecal coliform concentration. *Water Research*, *42*(13), 3327–3334. https://doi.org/10.1016/j.watres.2008.04.011

Groves, E., & Donnelly, C. W. (2005). Comparison of 3M Petrifilm environmental *Listeria* plate vs. standard methods in detecting *Listeria* from environmental surfaces. In *IAFP Annual Meeting, Abstract*.

Guerra, M. M., McLauchlin, J., & Bernardo, F. A. (2001). *Listeria* in ready-to-eat and unprocessed foods produced in Portugal. *Food Microbiology*, *18*(4), 423–429. https://doi.org/https://doi.org/10.1006/fmic.2001.0421

Gutiérrez-Rodríguez, E., Gundersen, A., Sbodio, A. O., & Suslow, T. V. (2012). Variable agronomic practices, cultivar, strain source and initial contamination dose differentially affect survival of *Escherichia coli* on spinach. *Journal of Applied Microbiology*, *112*(1), 109–118. https://doi.org/10.1111/j.1365-2672.2011.05184.x

Habann, M., Leiman, P. G., Vandersteegen, K., Van den Bossche, A., Lavigne, R., Shneider, M. M., … Klumpp, J. (2014). *Listeria* phage A511, a model for the contractile tail machineries of SPO1-related bacteriophages. *Molecular Microbiology*, *92*(1), 84–99. https://doi.org/10.1111/mmi.12539

Haeghebaert, S., Sulem, P., Deroudille, L., Vanneroy-Adenot, E., Bagnis, O., Bouvet, P., … Vaillant, V. (2003). Two outbreaks of *Salmonella* Enteritidis phage type 8 linked to the consumption of Cantal cheese made with raw milk, France, 2001. *Eurosurveillance*, *8*(7), 151–156. https://doi.org/https://doi.org/10.2807/esm.08.07.00419-en

Hagens, S., & Loessner, M. J. (2014). Phages of *Listeria* offer novel tools for diagnostics and biocontrol. *Frontiers in Microbiology*, *5*, 159. https://doi.org/10.3389/fmicb.2014.00159

Hamama, A., El Hankouri, N., & El Ayadi, M. (2002). Fate of enterotoxigenic *Staphylococcus aureus* in the presence of nisin-producing Lactococcus lactis strain during manufacture of Jben, a Moroccan traditional fresh cheese. *International Dairy Journal*, *12*(11), 933–938. https://doi.org/https://doi.org/10.1016/S0958-6946(02)00113-9

Hammer, P., Bockelmann, W., & Hoffmann, W. (2017). Fate of *Listeria* innocua during production and ripening of smeared hard cheese made from raw milk. *Journal of Dairy Science*, *100*(10), 7846–7856. https://doi.org/10.3168/jds.2017-12823

Hammerling, M. J., Gollihar, J., Mortensen, C., Alnahhas, R. N., Ellington, A. D., & Barrick, J. E. (2016). Expanded Genetic Codes Create New Mutational Routes to Rifampicin Resistance in *Escherichia coli*. *Molecular Biology and Evolution*, *33*(8), 2054–2063. https://doi.org/10.1093/molbev/msw094

Hancock, D., Besser, T., Lejeune, J., Davis, M., & Rice, D. (2001). The control of VTEC in the animal reservoir. *International Journal of Food Microbiology*, *66*(1–2), 71–78. https://doi.org/10.1016/S0168-1605(00)00487-6

Haran, K. P., Godden, S. M., Boxrud, D., Jawahir, S., Bender, J. B., & Sreevatsan, S. (2012). Prevalence and characterization of *Staphylococcus aureus*, including methicillin-resistant *Staphylococcus aureus*, isolated from bulk tank milk from Minnesota dairy farms. *Journal of Clinical Microbiology*, *50*(3), 688–695. https://doi.org/10.1128/JCM.05214-11

Hargrove, R. E., McDonough, F. E., & Mattingly, W. A. (1969). Factors Affecting Survival Of *Salmonella* In Cheddar And Colby Cheese. *Journal of Milk and Food Technology*, *32*(12), 480–484. https://doi.org/10.4315/0022-2747-32.12.480

Harris, L. J., Bender, J., Bihn, E. A., Blessington, T., Danyluk, M. D., Delaquis, P., …

Suslow, T. V. (2012). A Framework For Developing Research Protocols For Evaluation Of Microbial Hazards And Controls during Production That Pertain to the Quality of Agricultural Water Contacting Fresh Produce That May Be Consumed Raw. *Journal of Food Protection*, *75*(12), 2251–2273. https://doi.org/10.4315/0362-028X.JFP-12-252

Harris, L. J., Farber, J. N., Beuchat, L. R., Parish, M. E., Suslow, T. V., Garrett, E. H., & Busta, F. F. (2003). Outbreaks associated with fresh produce: incidence, growth and survival of pathogens in fresh and fresh-cut produce. *Comprehensive Reviews in Food Science and Food Safety*, *2*(s1), 78–141. https://doi.org/10.1111/j.1541-4337.2003.tb00031.x

Harter, E., Wagner, E. M., Zaiser, A., Halecker, S., Wagner, M., & Rychli, K. (2017). Stress survival islet 2, predominantly present in *Listeria monocytogenes* strains of sequence type 121, is involved in the alkaline and oxidative stress responses. *Applied and Environmental Microbiology*, *83*(16). https://doi.org/10.1128/AEM.00827-17

Headrick, M. L., Korangy, S., Bean, N. H., Angulo, F. J., Altekruse, S. F., Potter, M. E., & Klontz, K. C. (1998). The epidemiology of raw milk-associated foodborne disease outbreaks reported in the United States, 1973 through 1992. *American Journal of Public Health*, *88*(8), 1219–1221. https://doi.org/10.2105/AJPH.88.8.1219

Health Canada. (2015). Canadian Dairy Information Centre; National Dairy Code. http://www.dairyinfo.gc.ca/index_e.php?s1=dr-rl&s2=canada&s3=ndc-cnpl&s4=05-2005. Accessed 11.24.2016.

Health Canada and U.S. Food and Drug Administration (FDA) (2015). Joint FDA/Health Canada Quantitative Assessment of the Risk of Listeriosis from Soft-Ripened Cheese Consumption in the United States and Canada. https://www.fda.gov/downloads/Food/FoodScienceResearch/RiskSafetyAssessment/UCM429419.pdf

Health Protection Agency (HPA). (2009). Guidelines for Assessing the Microbiological Safety of Ready-to-Eat Foods Placed on the Market. *Health Protection Agency, London.*, (November), 33.

Hedberg, C., Korlath, J., D'Aoust, J., & ET, A. (1992). A multistate outbreak of *Salmonella* javiana and *Salmonella* oranienburg infections due to consumption of contaminated cheese. *JAMA*, *268*(22), 3203–3207. Retrieved from http://dx.doi.org/10.1001/jama.1992.03490220047026

Heiman, K. E., Mody, R. K., Johnson, S. D., Griffin, P. M., & Gould, L. H. (2015). *Escherichia coli* O157 Outbreaks in the United States , 2003 – 2012. *Emerg Infect Dis*, *21*(8), 1293–1301. https://doi.org/10.3201/eid2108.141364

Heuvelink, A. E., Bleumink, B., Van Den Biggelaar, F. L. A. M., Te Giffel, M. C., Beumer, R. R., & De Boer2, E. (1998). Occurrence and Survival of Verocytotoxin-Producing *Escherichia coli* 0157 in Raw Cow's Milk in the Netherlands. *Journal of Food Protection*, *61*(12), 1597–1601. https://doi.org/10.4315/0362-028X-61.12.1597

Hill, B., Smythe, B., Lindsay, D., & Shepherd, J. (2012). Microbiology of raw milk in New Zealand. *International Journal of Food Microbiology*, *157*(2), 305–308. https://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2012.03.031

Hirneisen, K. A., Sharma, M., & Kniel, K. E. (2012). Human Enteric Pathogen

Internalization by Root Uptake into Food Crops. *Foodborne Pathogens and Disease*, *9*(5), 396–405. https://doi.org/10.1089/fpd.2011.1044

Ho, A. J., Lappi, V. R., & Wiedmann, M. (2007). Longitudinal Monitoring of *Listeria monocytogenes* Contamination Patterns in a Farmstead Dairy Processing Facility. *Journal of Dairy Science*, *90*(5), 2517–2524. https://doi.org/10.3168/jds.2006-392

Ho, J., Shands, K., Friedland, G., Eckind, P., & Fraser, D. (1986). An outbreak of type 4b *Listeria monocytogenes* infection involving patients from eight boston hospitals. *Archives of Internal Medicine*, *146*(3), 520–524. Retrieved from http://dx.doi.org/10.1001/archinte.1986.00360150134016

Hodgson, D. A. (2002). Generalized transduction of serotype 1/2 and serotype 4b strains of *Listeria monocytogenes*. *Molecular Microbiology*, *35*(2), 312–323. https://doi.org/10.1046/j.1365-2958.2000.01643.x

Holt, P. S., Buhr, R. J., Cunningham, D. L., & Porter R. E., J. R. (1994). Effect of Two Different Molting Procedures on a *Salmonella* enteritidis Infection. *Poultry Science*, *73*(8), 1267–1275. Retrieved from http://dx.doi.org/10.3382/ps.0731267

Honish, L., Predy, G., Hislop, N., Kowalewska-grochowska, K., Trottier, L., Kreplin, C., & Zazulak, I. (2005). An Outbreak of *E. coli* O157:H7 Haemorrhagic colitis Associated with Unpasteurized Gouda Cheese. *Canadian Journal of Public Health*, *96*(3), 182–184.

Horter, B., & Lubrant, H. (2004). 3M Petrifilm Environmental *Listeria* plate for rapid enumeration of *Listeria* from environmental surfaces. In *IAFP Annual Meeting* (p. P138).

Hu, L., Robert, C. A. M., Cadot, S., Zhang, X., Ye, M., Li, B., Manzo, D., Chervet, N., Steinger., Van Der H., Marcel G.A., Schlaeppi, K., Erb M. (2018). Root exudate metabolites drive plant-soil feedbacks on growth and defense by shaping the rhizosphere microbiota. *Nature Communications*, *9*(1), 1–13. https://doi.org/10.1038/s41467-018-05122-7

Hudson, L. M., Chen, J., Hill, A. R., & Griffiths, M. W. (1997). Bioluminescence: A rapid indicator of *Escherichia coli* O157:H7 in selected yogurt and cheese varieties. *Journal Of Food Protection*, *60*(8), 891–897. https://doi.org/10.4315/0362-028X-60.8.891

Hughes, J. M., Wilson, M. E., Johnson, K. E., Thorpe, C. M., & Sears, C. L. (2006). The Emerging Clinical Importance of Non-O157 Shiga Toxin--Producing *Escherichia coli*. *Clinical Infectious Diseases*, *43*(12), 1587–1595. https://doi.org/10.1086/509573

Hussein, H. S., & Sakuma, T. (2005). Invited Review: Prevalence of Shiga Toxin-Producing *Escherichia coli* in Dairy Cattle and Their Products. *Journal of Dairy Science*, *88*(2), 450–465. https://doi.org/10.3168/jds.S0022-0302(05)72706-5

Hutchison, M. L., Walters, L. D., Moore, A., Crookes, K. M., & Avery, S. M. (2004). Effect of Length of Time before Incorporation on Survival of Pathogenic Bacteria Present in Livestock Wastes Applied to Agricultural Soil Effect of Length of Time before Incorporation on Survival of Pathogenic Bacteria Present in Livestock Wastes Applied. *Applied and Environmental Microbiology*, *70*(9), 5111–5118. https://doi.org/10.1128/AEM.70.9.5111

ICMSF (1996) Intestinally pathogenic *Escherichia coli* Ch 7 In: Microorganisms in food 5: Microbiological specifications of food pathogens. Blackie Academic and

Professional, London, p. 126–140
Ingram, D. T., Patel, J., & Sharma, M. (2011). Effect of Repeated Irrigation with Water Containing Varying Levels of Total Organic Carbon on the Persistence of *Escherichia coli* O157:H7 on Baby Spinach. *Journal of Food Protection*, *74*(5), 709–717. http://doi.org/10.4315/0362-028X.JFP-10-426.
Institute for Trade and Commercial Diplomacy (ITCD). (1999) Codex Alimentarius Commission. http://www.commercialdiplomacy.org/ma_projects/ma_dairy_hygiene2.htm. htm#_ftn27. Accessed 8.8.2015.
International Commission of Microbiological Specifications of Foods (ICMSF). (1986). Microorganisms in Foods Book 2: Use of Data for Assessing Process Control and Product Acceptance. http://www.icmsf.org/publications/books.html. Accessed 6.22.2015.
International Dairy Federation (IDF). (2016). *Escherichia coli* as indicator in cheese processing. http://www.fil-idf.org/wp-content/uploads/2016/12/Escherichia-coli-as-indicators-in-cheese-processing.pdf. Accessed 2.8.2017.
Islam, M., Doyle, M. P., Phatak, S. C., Millner, P., & Jiang, X. (2005). Survival of *Escherichia coli* O157:H7 in soil and on carrots and onions grown in fields treated with contaminated manure composts or irrigation water. *Food Microbiology*, *22*(1), 63–70. https://doi.org/10.1016/j.fm.2004.04.007
Islam, M., Morgan, J., Doyle, M. P., & Jiang, X. (2004). Fate Of *Escherichia coli* O157:H7 In Manure Compost–Amended Soil And On Carrots And Onions Grown In An Environmentally Controlled Growth Chamber. *Journal of Food Protection*, *67*(3), 574–578. https://doi.org/10.4315/0362-028X-67.3.574
Islam, M., Morgan, J., Doyle, M. P., Phatak, S. C., Millner, P., & Jiang, X. (2004). Persistence of *Salmonella enterica* Serovar Typhimurium on Lettuce and Parsley and in Soils on Which They Were Grown in Fields Treated with Contaminated Manure Composts or Irrigation Water. *Foodborne Pathogens and Disease*, *1*(1), 27–35. https://doi.org/10.1089/153531404772914437
Ismail, R., Aviat, F., Gay-Perret, P., Le Bayon, I., Federighi, M., & Michel, V. (2017). An assessment of *L. monocytogenes* transfer from wooden ripening shelves to cheeses: Comparison with glass and plastic surfaces. *Food Control*, *73*, 273–280. https://doi.org/10.1016/j.foodcont.2016.08.014
Ismaïl, R., Aviat, F., Michel, V., Le Bayon, I., Gay-Perret, P., Kutnik, M., & Fédérighi, M. (2013). Methods for recovering microorganisms from solid surfaces used in the food industry: A review of the literature. *International Journal of Environmental Research and Public Health*, *10*(11), 6169–6183. https://doi.org/10.3390/ijerph10116169
Ismaïl, R., Le Bayon, I., Michel, V., Jequel, M., Kutnik, M., Aviat, F., & Fédérighi, M. (2015). Comparative Study of Three Methods for Recovering Microorganisms from Wooden Surfaces in the Food Industry. *Food Analytical Methods*, *8*(5), 1238–1247. https://doi.org/10.1007/s12161-014-0008-3
Iturriaga, M. H., Escartín, E. F., Beuchat, L. R., & Martínez-Peniche, R. (2003). Effect od inoculum size, relative humidity, storage temperature, and ripening stage on the attachment of *Salmonella* Montevideo to tomatoes and tomatillos. *Journal of Food Protection*, *66*(10), 1756–1761. https://doi.org/10.4315/0362-028X-66.10.1756

Jablasone, J., Warriner, K., & Griffiths, M. (2005). Interactions of *Escherichia coli* O157:H7, *Salmonella* typhimurium and *Listeria monocytogenes* plants cultivated in a gnotobiotic system. *International Journal of Food Microbiology*, *99*(1), 7–18. https://doi.org/10.1016/j.ijfoodmicro.2004.06.011

Jacquet, C., Doumith, M., Gordon, J. I., Martin, P. M. V, Cossart, P., & Lecuit, M. (2004). A molecular marker for evaluating the pathogenic potential of foodborne *Listeria monocytogenes*. *J Infect Dis.*, *189*(11), 2094–2100. https://doi.org/10.1086/420853

Jamieson, R. C., Gordon, R. J., Sharples, K. E., Stratton, G. W., & Madani, A. (2002). Movement and persistence of fecal bacteria in agricultural soils and subsurface drainage water: A review. *Canadian Biosystems Engineering*, *44*, 1.1-1.9.

Jayarao, B. M., Donaldson, S. C., Straley, B. a, Sawant, a a, Hegde, N. V, & Brown, J. L. (2006). A survey of foodborne pathogens in bulk tank milk and raw milk consumption among farm families in pennsylvania. *Journal of Dairy Science*, *89*(7), 2451–2458. https://doi.org/10.3168/jds.S0022-0302(06)72318-9

Jayarao, B. M., & Henning, D. R. (2001). Prevalence of Foodborne Pathogens in Bulk Tank Milk. *Journal of Dairy Science*, *84*(10), 2157–2162. https://doi.org/10.3168/jds.S0022-0302(01)74661-9

Jeffers, G. T., Bruce, J. L., McDonough, P. L., Scarlett, J., Boor, K. J., & Wiedmann, M. (2001). Comparative genetic characterization of *Listeria monocytogenes* isolates from human and animal listeriosis cases. *Microbiology (Reading, England)*, *147*(Pt 5), 1095–1104. https://doi.org/10.1099/00221287-147-5-1095

Jiang, X., Morgan, J., & Doyle, M. P. (2002). Fate of *Escherichia coli* O157: H7 in Manure-Amended Soil. *Applied and Environmental Microbiology*, *68*(5), 2605–2609. https://doi.org/10.1128/AEM.68.5.2605

Jinneman, K. C. K., Yoshitomi, K. J., & Weagant, S. D. (2003). Multiplex real-time PCR method to identify Shiga toxin genes stx1 and stx2 and *Escherichia coli* O157: H7/H− serotype. *Applied and …*, *69*(10), 6327–6333. https://doi.org/10.1128/AEM.69.10.6327

Johnson, M. E. (2013). Mesophilic and Thermophilic Cultures Used in Traditional Cheesemaking. *Microbiology Spectrum*, *1*(1), CM-0004-2012. https://doi.org/10.1128/microbiolspec.CM-0004-2012.Correspondence

Jordan, K. N., & Davies, K. W. (2001). Sodium chloride enhances recovery and growth of acid-stressed *E. coli* O157:H7. *Letters in Applied Microbiology*, *32*(5), 312–315. https://doi.org/10.1046/j.1472-765X.2001.00911.x

Jørgensen, H. J., Mork, T., Høgåsen, H. R., & Rørvik, L. M. (2005). Enterotoxigenic *Staphylococcus aureus* in bulk milk in Norway. *Journal of Applied Microbiology*, *99*(1), 158–166. https://doi.org/10.1111/j.1365-2672.2005.02569.x

Kabuki, D. Y., Kuaye, A. Y., Wiedmann, M., & Boor, K. J. (2004). Molecular Subtyping and Tracking of *Listeria monocytogenes* in Latin-Style Fresh-Cheese Processing Plants. *Journal of Dairy Science*, *87*(9), 2803–2812. https://doi.org/10.3168/jds.S0022-0302(04)73408-6

Kadivar, H., & Stapleton, A. E. (2003). Ultraviolet radiation alters maize phyllosphere bacterial diversity. *Microbial Ecology*, *45*(4), 353–361. https://doi.org/10.1007/s00248-002-1065-5

Kaper, J. B., & Sperandio, V. (2005). Bacterial Cell-to-Cell Signaling in the

Gastrointestinal Tract MINIREVIEW Bacterial Cell-to-Cell Signaling in the Gastrointestinal Tract. *Infection and Immunity*, *73*(6), 3197–3209. https://doi.org/10.1128/IAI.73.6.3197

Karns, J. S., Van Kessel, J. S., McCluskey, B. J., & Perdue, M. L. (2005). Prevalence of *Salmonella* enterica in Bulk Tank Milk from US Dairies as Determined by Polymerase Chain Reaction*. *Journal of Dairy Science*, *88*(10), 3475–3479. https://doi.org/https://doi.org/10.3168/jds.S0022-0302(05)73031-9

Kathariou, S. (2002). *Listeria monocytogenes* virulence and pathogenicity, a food safety perspective. *Journal of Food Protection*, *65*(11), 1811–1829. https://doi.org/10.4315/0362-028X-65.11.1811

Kelleher, B. P., Leahy, J. J., Henihan, a M., O'Dwyer, T. F., Sutton, D., & Leahy, M. J. (2002). Advances in poultry litter disposal technology--a review. *Bioresource Technology*, *83*(1), 27–36. https://doi.org/10.1016/S0960-8524(01)00133-X

Kells, J., & Gilmour, A. (2004). Incidence of *Listeria monocytogenes* in two milk processing environments, and assessment of *Listeria monocytogenes* blood agar for isolation. *International Journal of Food Microbiology*, *91*(2), 167–174. https://doi.org/10.1016/S0168-1605(03)00378-7

Khelef, N., Lecuit, M., Buchrieser, C., Cabanes, D., Dussurget, O., & Cossart, P. (2006). *Listeria monocytogenes* and the genus Listeria. In *The Prokaryotes* (pp. 404–476). Springer.

Kindstedt, P. S. (2013). The Basics of Cheesemaking. *Microbiology Spectrum*, *1*(May 2016), CM-0002-2012. https://doi.org/10.1128/microbiolspec.CM-0002-2012.Correspondence

Knief, C., Delmotte, N., Chaffron, S., Stark, M., Innerebner, G., Wassmann, R., … Vorholt, J. A. (2011). Metaproteogenomic analysis of microbial communities in the phyllosphere and rhizosphere of rice. *The Isme Journal*, *6*, 1378. Retrieved from http://dx.doi.org/10.1038/ismej.2011.192

Kõljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AFS, Bahram M, Bates ST, Bruns TD, Bengtsson-Palme J, Callaghan TM, Douglas B (2013). Towards a unified paradigm for sequence-based identification of fungi. Mol Ecol 22: 5271−5277

Kovačević, J., Mesak, L. R., & Allen, K. J. (2012). Occurrence and characterization of *Listeria* spp. in ready-to-eat retail foods from Vancouver, British Columbia. *Food Microbiology*, *30*(2), 372–378. https://doi.org/10.1016/j.fm.2011.12.015

Kudva, I. T., Blanch, K., & Hovde, C. J. (1998). Analysis of *Escherichia coli* O157 : H7 Survival in Ovine or Bovine Manure and Manure Slurry Analysis of *Escherichia coli* O157 : H7 Survival in Ovine or Bovine Manure and Manure Slurry, *64*(9), 3166–3174.

Kremer, P. H. C., Lees, J. A., Koopmans, M. M., Ferwerda, B., Arends, A. W. M., Feller, M. M., & Bentley, S. D. (2017). Benzalkonium tolerance genes and outcome in *Listeria monocytogenes* meningitis. *Clinical Microbiology and Infection*, *23*(4), 265.e1-265.e7. https://doi.org/10.1016/J.CMI.2016.12.008

Kusumaningrum, H. D., Van Putten, M. M., Rombouts, F. M., & Beumer, R. R. (2002). Effects of antibacterial dishwashing liquid on foodborne pathogens and competitive microorganisms in kitchen sponges. *Journal of Food Protection*, *65*(1), 61–65. https://doi.org/10.4315/0362-028X-65.1.61

Lahou, E., & Uyttendaele, M. (2014). Evaluation of Three Swabbing Devices for Detection of *Listeria monocytogenes* on Different Types of Food Contact Surfaces, 804–814. https://doi.org/10.3390/ijerph110100804

Lahou, E., & Uyttendaele, M. (2017). Growth potential of *Listeria monocytogenes* in soft, semi-soft and semi-hard artisanal cheeses after post-processing contamination in deli retail establishments. *Food Control*, *76*, 13–23. https://doi.org/10.1016/j.foodcont.2016.12.033

Larsen, M. H., Dalmasso, M., Ingmer, H., Langsrud, S., Malakauskas, M., Mader, A., … Jordan, K. (2014). Persistence of foodborne pathogens and their control in primary and secondary food production chains. *Food Control*, *44*, 92–109. https://doi.org/10.1016/j.foodcont.2014.03.039

Larson, A. E., Johnson, E. A., & Nelson, J. H. (1999). Survival of *Listeria monocytogenes* in Commercial Cheese Brines. *Journal of Dairy Science*, *82*(9), 1860–1868. https://doi.org/10.3168/jds.S0022-0302(99)75419-6

Lau, M. M., & Ingham, S. C. (2001). Survival of faecal indicator bacteria in bovine manure incorporated into soil. *Letters in Applied Microbiology*, *33*(2), 131–136. http://doi.org/10.1046/j.1472-765X.2001.00962.x

Lauber, C. L., Hamady, M., Knight, R., & Fierer, N. (2009). Pyrosequencing-based assessment of soil pH as a predictor of soil bacterial community structure at the continental scale. *Applied and Environmental Microbiology*, *75*(15), 5111–5120. https://doi.org/10.1128/AEM.00335-09

Law, D. (2000). Virulence factors of *Escherichia coli* O157 and other Shiga toxin-producing *E. coli*. *Journal of Applied Microbiology*, *88*(5), 729–745. https://doi.org/10.1046/j.1365-2672.2000.01031.x

Lee, S. K., Lee, H. J., Min, H. Y., Park, E. J., Lee, K. M., Ahn, Y. H., … Pyee, J. H. (2005). Antibacterial and antifungal activity of pinosylvin, a constituent of pine. *Fitoterapia*, *76*(2), 258–260. https://doi.org/10.1016/j.fitote.2004.12.004

Lekkas, P. (2016). *The Microbial Ecology Of Listeria monocytogenes As Impacted By Three Environments: A Cheese Microbial Community ; A Farm Environment ; And A Soil Microbial Community; Chapter 4:* Fate Of *Escherichia coli* And *Listeria* Species In Dairy Manure Amendments Applied In Soils In The Northeastern United States. Thesis.

Le Loir Y., Baron F., and Gautier M. (2003). *Staphylococcus aureus* and food poisoning. *Genet. Mol. Res*. 2 (1): 63-76.

Lemon, K. P., Higgins, D. E., & Kolter, R. (2007). Flagellar motility is critical for *Listeria monocytogenes* biofilm formation. *Journal of Bacteriology*, *189*(12), 4418–4424. https://doi.org/10.1128/JB.01967-06

Lemunier, M., Francou, C., Rousseaux, S., Houot, S., Dantigny, P., Piveteau, P., & Guzzo, J. (2005). Long-term survival of pathogenic and sanitation indicator bacteria in experimental biowaste composts. *Applied and Environmental Microbiology*, *71*(10), 5779–5786. https://doi.org/10.1128/AEM.71.10.5779-5786.2005

Leyer, G. J., & Johnson, E. A. (1992). Acid adaptation promotes survival of *Salmonella* spp. in cheese. *Applied and Environmental Microbiology*, *58*(6), 2075 LP-2080. Retrieved from http://aem.asm.org/content/58/6/2075.abstract

Leyer, G. J., & Johnson, E. a. (1993). Acid adaptation induces cross-protection against environmental stresses in *Salmonella* typhimurium . Acid Adaptation Induces Cross-

Protection against Environmental Stresses in *Salmonella* typhimurium, *59*(6), 1842–1847.
Licitra, G., Lortal, S., & Valence, F. (2014). Wooden Tools: Reservoirs of Microbial Biodiversity in Traditional Cheesemaking. *Microbiology Spectrum*, *2*(1), 1–8. https://doi.org/10.1128/microbiolspec.CM-0008-2012
Lin, A., Kase, J. A., & Moore, M. M. (n.d.). STEC Molecular Serotyping Protocol. https://www.fda.gov/downloads/food/foodscienceresearch/laboratorymethods/ucm360921.pdf. Accessed November 29, 2018.
Lin, A., Kase, J. A., Moore, M. M., Son, I., Tran, N., Clotilde, L. M., … Hammack, T. S. (2013). Multilaboratory Validation of a Luminex Microbead-Based Suspension Array for the Identification of the 11 Most Clinically Relevant Shiga Toxin–Producing *Escherichia coli* O Serogroups. *Journal of Food Protection*, *76*(5), 867–870. https://doi.org/10.4315/0362-028X.JFP-12-468
Lin, A., Sultan, O., Lau, H. K., Wong, E., Hartman, G., & Lauzon, C. R. (2011). O serogroup specific real time PCR assays for the detection and identification of nine clinically relevant non-O157 STECs. *Food Microbiology*, *28*(3), 478–483. https://doi.org/10.1016/j.fm.2010.10.007
Lira, W. M., Macedo, C., & Marin, J. M. (2004). The incidence of Shiga toxin-producing *Escherichia coli* in cattle with mastitis in Brazil. *Journal of Applied Microbiology*, *97*(4), 861–866. https://doi.org/10.1111/j.1365-2672.2004.02384.x
Little, C. L., Rhoades, J. R., Sagoo, S. K., Harris, J., Greenwood, M., Mithani, V., … McLauchlin, J. (2008). Microbiological quality of retail cheeses made from raw, thermized or pasteurized milk in the UK. *Food Microbiology*, *25*(2), 304–312. https://doi.org/10.1016/j.fm.2007.10.007
Locatelli A, Spor A, Jolivet C, Piveteau P, Hartmann A (2013) Biotic and Abiotic Soil Properties Influence Survival of *Listeria monocytogenes* in Soil. PLOS ONE 8(10): e75969. https://doi.org/10.1371/journal.pone.0075969
Loessner, M. J., Rees, C. E., Stewart, G. S., & Scherer, S. (1996). Construction of luciferase reporter bacteriophage A511::luxAB for rapid and sensitive detection of viable *Listeria* cells. *Applied and Environmental Microbiology*, *62*(4), 1133 LP-1140. Retrieved from http://aem.asm.org/content/62/4/1133.abstract
Loessner, M. J., Wendlinger, G., & Scherer, S. (1995). Heterogeneous endolysins in *Listeria monocytogenes* bacteriophages: a new class of enzymes and evidence for conserved holin genes within the siphoviral lysis cassettes. *Molecular Microbiology*, *16*(6), 1231–1241. https://doi.org/10.1111/j.1365-2958.1995.tb02345.x
Loir, Y. Le, Baron, F., & Gautier, M. (2003). *Staphylococcus aureus* and food poisoning, *2*(1), 63–76.
Lomonaco, S., Verghese, B., Gerner-Smidt, P., Tarr, C., Gladney, L., Joseph, L., … Knabel, S. (2013). Novel Epidemic Clones of *Listeria monocytogenes*, United States, 2011. *Emerging Infectious Diseases*, *19*(1), 147–150. Retrieved from http://search.ebscohost.com/login.aspx?direct=true&db=eih&AN=84519141&site=ehost-live
Lourenço, A., Neves, E., & Brito, L. (2009). Susceptibility of *Listeria monocytogenes* from traditional cheese-dairies to in-use sanitizers. *Food Control*, *20*(6), 585–589. https://doi.org/10.1016/j.foodcont.2008.08.009
Low, J. C., & Donachie, W. (1997). A review of *Listeria monocytogenes* and listeriosis.

Veterinary Journal, *153*(1), 9–29. https://doi.org/10.1016/S1090-0233(97)80005-6
Lowry R. (2016). VassarStats: Website for Statistical Computation. http://vassarstats.net/. Accessed 10.3.2016.
Lücke, F. K., & Skowyrska, A. (2015). Hygienic aspects of using wooden and plastic cutting boards, assessed in laboratory and small gastronomy units. *Journal Fur Verbraucherschutz Und Lebensmittelsicherheit*, *10*(4), 317–322. https://doi.org/10.1007/s00003-015-0949-5
Lukinmaa, S., Aarnisalo, K., Suihko, M. L., & Siitonen, A. (2004). Diversity of *Listeria monocytogenes* isolates of human and food origin studied by serotyping, automated ribotyping and pulsed-field gel electrophoresis. *Clinical Microbiology and Infection*, *10*(6), 562–568. https://doi.org/10.1111/j.1469-0691.2004.00876.x
Lundén, J., Tolvanen, R., & Korkeala, H. (2004). Human Listeriosis Outbreaks Linked to Dairy Products in Europe. *Journal of Dairy Science*, *87*(Table 1), E6–E12. https://doi.org/10.3168/jds.S0022-0302(04)70056-9
Luo, C., Walk, S. T., Gordon, D. M., Feldgarden, M., Tiedje, J. M., & Konstantinidis, K. T. (2011). Genome sequencing of environmental *Escherichia coli* expands understanding of the ecology and speciation of the model bacterial species. *Proceedings of the National Academy of Sciences*, *108*(17), 7200–7205. https://doi.org/10.1073/pnas.1015622108
Ma, L., Zhang, G., & Doyle, M. P. (2011). Green fluorescent protein labeling of Listeria, *Salmonella*, and *Escherichia coli* O157:H7 for safety-related studies. *PLoS ONE*, *6*(4), 1–7. https://doi.org/10.1371/journal.pone.0018083
MacDonald, P. D. M., Whitwam, R. E., Boggs, J. D., MacCormack, J. N., Anderson, K. L., Reardon, J. W., … Sobel, J. (2005). Outbreak of Listeriosis among Mexican Immigrants as a Result of Consumption of Illicitly Produced Mexican-Style Cheese. *Clinical Infectious Diseases*, *40*(5), 677–682. https://doi.org/10.1086/427803
Maguire, H., Cowden, J., Jacob, M., Rowe, B., Roberts, D., Bruce, J., & Mitchell, E. (1992). An outbreak of *Salmonella* dublin infection in England and Wales associated with a soft unpasteurized cows' milk cheese. *Epidemiology and Infection*, *109*(3), 389–396. https://doi.org/10.1017/S0950268800050378
Maher, M. M., Jordan, K. N., Upton, M. E., & Coffey, A. (2001). Growth and survival of *E. coli* O157:H7 during the manufacture and ripening of a smear-ripened cheese produced from raw milk. *Journal of Applied Microbiology*, *90*(2), 201–207. https://doi.org/10.1046/j.1365-2672.2001.01232.x
Malley, T. J. V, Butts, J., & Wiedmann, M. (2015). Seek and destroy process: *Listeria monocytogenes* process controls in the ready-to-eat meat and poultry industry. *Journal of Food Protection*, *78*(2), 436–445. https://doi.org/10.4315/0362-028X.JFP-13-507
Manan Sharma and Russell Reynnells. (2016). Importance of Soil Amendments: Survival of Bacterial Pathogens in Manure and Compost Used as Organic Fertilizers. *Microbiology Spectrum*, *4*(4), 1–13. https://doi.org/doi:10.1128/microbiolspec.PFS-0010-2015
Manning, S. D., Motiwala, A. S., Springman, A. C., Qi, W., Lacher, D. W., Ouellette, L. M., … Whittam, T. S. (2008). Variation in virulence among clades of *Escherichia coli* O157:H7 associated with disease outbreaks. *Proceedings of the National Academy of Sciences*, *105*(12), 4868–4873.

https://doi.org/10.1073/pnas.0710834105
Mariani, C., Briandet, R., Chamba, J.-F., Notz, E., Carnet-Pantiez, A., Eyoug, R. N., & Oulahal, N. (2007). Biofilm Ecology of Wooden Shelves Used in Ripening the French Raw Milk Smear Cheese Reblochon de Savoie. *Journal of Dairy Science*, *90*(4), 1653–1661. https://doi.org/10.3168/JDS.2006-190
Mariani, C., Oulahal, N., Chamba, J. F., Dubois-Brissonnet, Notz, E., & Briandet, R. (2011). Inhibition of *Listeria monocytogenes* by resident biofilms present on wooden shelves used for cheese ripening. *Food Control*, *22*(8), 1357–1362. https://doi.org/10.1016/j.foodcont.2011.02.012
Marielle Gay, & Albert Amgar. (2005). Factors moderating *Listeria monocytogenes* growth in raw milk and in soft cheese made from raw milk. *Lait*, *85*(3), 153–170. https://doi.org/10.1051/lait:2005010
Mark Ibekwe, A., Grieve, C. M., Papiernik, S. K., & Yang, C. H. (2009). Persistence of *Escherichia coli* O157:H7 on the rhizosphere and phyllosphere of lettuce. *Letters in Applied Microbiology*, *49*(6), 784–790. https://doi.org/10.1111/j.1472-765X.2009.02745.x
Markland, S. M., Shortlidge, K. L., Hoover, D. G., Yaron, S., Patel, J., Singh, A., … Kniel, K. E. (2013). Survival of Pathogenic *Escherichia coli* on Basil, Lettuce, and Spinach. *Zoonoses and Public Health*, *60*(8), 563–571. https://doi.org/10.1111/zph.12033
Marouani-Gadri, N., Chassaing, D., & Carpentier, B. (2009). Comparative Evaluation Of Biofilm Formation And Tolerance To A Chemical Shock Of Pathogenic and Nonpathogenic *Escherichia coli* O157:H7 Strains. *Journal of Food Protection*, *72*(1), 157–164. https://doi.org/10.4315/0362-028X-72.1.157
Marouani-Gadri, N., Firmesse, O., Chassaing, D., Sandris-Nielsen, D., Arneborg, N., & Carpentier, B. (2010). Potential of *Escherichia coli* O157:H7 to persist and form viable but non-culturable cells on a food-contact surface subjected to cycles of soiling and chemical treatment. *International Journal of Food Microbiology*, *144*(1), 96–103. https://doi.org/10.1016/j.ijfoodmicro.2010.09.002
Martin, N. H., Trmčič, A., Hsieh, T. H., Boor, K. J., & Wiedmann, M. (2016). The evolving role of coliforms as indicators of unhygienic processing conditions in dairy foods. *Frontiers in Microbiology*, *7*(SEP), 1–8. https://doi.org/10.3389/fmicb.2016.01549
Maurel, B., Mastracci, T., Spear, R., Hertault, A., Azzaoui, R., Sobocinski, J., & Haulon, S. 2016. Branched and fenestrated options to treat aortic arch aneurysms. *Journal of Cardiovascular Sugery (Torino), 57*, 686–697. https://doi.org/10.1128/AEM.70.8.4458
McCollum, J. T., Cronquist, A. B., Silk, B. J., Jackson, K. A., O'Connor, K. A., Cosgrove, S., … Mahon, B. E. (2013). Multistate Outbreak of Listeriosis Associated with Cantaloupe. *New England Journal of Medicine*, *369*(10), 944–953. https://doi.org/10.1056/NEJMoa1215837
Mccollum, J. T., WILLIAMS, N. J., BEAM, S. W., COSGROVE, S., ETTESTAD, P. J., GHOSH, T. S., … CRONQUIST, A. B. (2012). Multistate Outbreak of *Escherichia coli* O157:H7 Infections Associated with In-Store Sampling of an Aged Raw-Milk Gouda Cheese, 2010. *Journal of Food Protection*, *75*(10), 1759–1765. https://doi.org/10.4315/0362-028X.JFP-12-136

McDonald, D., Price, M. N., Goodrich, J., Nawrocki, E. P., Desantis, T. Z., Probst, A., Andersen G.L., Knight R., Hugenholtz, P. (2011). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *ISME Journal*, *6*(3), 610–618. http://doi.org/10.1038/ismej.2011.139

McDonough, P. L., Rossiter, C. A., Rebhun, R. B., Stehman, S. M., Lein, D. H., & Shin, S. J. (2000). Prevalence of *Escherichia coli* O157 : H7 from cull dairy cows in New York State and comparison of culture methods used during preharvest food safety investigations. *Journal of Clinical Microbiology*, *38 (1)*(1), 318–322.

McKellar, R. C., Lu, X., & Delaquis, P. J. (2002). A probability model describing the interface between survival and death of *Escherichia coli* O157:H7 in a mayonnaise model system. *Food Microbiology*, *19*(2–3), 235–247. https://doi.org/10.1006/fmic.2001.0449

McLauchlin, J., Greenwood, M. H., & Pini, P. N. (1990). The occurrence of *Listeria monocytogenes* in cheese from a manufacturer associated with a case of listeriosis. *International Journal of Food Microbiology*, *10*(3–4), 255–262. https://doi.org/10.1016/0168-1605(90)90073-E

McLaughlin, H. P., Casey, P. G., Cotter, J., Gahan, C. G. M., & Hill, C. (2011). Factors affecting survival of *Listeria monocytogenes* and *Listeria* innocua in soil samples. *Archives of Microbiology*, *193*(11), 775–785. https://doi.org/10.1007/s00203-011-0716-7

Mcmanus, C., & LANIER, J. M. (1987). *Salmonella*, Campylobacter jejuni, and Yersinia enterocolitica in Raw Milk. *Journal of Food Protection*, *50*(1), 51–55. https://doi.org/10.4315/0362-028X-50.1.51

Mead, P. S., Slutsker, L., Dietz, V., McCaig, L. F., Bresee, J. S., Shapiro, C., … Tauxe, R. V. (1999). Food-related illness and death in the United States. *Emerging Infectious Diseases*, *5*(5), 607–625. https://doi.org/10.3201/eid0505.990502

Mehta, A., & Tatini, S. R. (1994). An Evaluation of the Microbiological Safety of Reduced-Fat Cheddar-like Cheese. *Journal of Food Protection*, *57*(9), 776–779. https://doi.org/10.4315/0362-028X-57.9.776

Melo, J., Andrew, P. W., & Faleiro, M. L. (2015). *Listeria monocytogenes* in cheese and the dairy environment remains a food safety challenge: The role of stress responses. *Food Research International*, *67*, 75–90. https://doi.org/10.1016/j.foodres.2014.10.031

Meyrand, A., Boutrand-Loei S., Ray-Gueniot S., Mazuy C., Gaspard, C.E., Jaubert G., Perrin G., Lapeyre C., Vernozy-Rozand C. 1998. (1998). Growth and enterotoxin production of *Staphylococcus aureus* during the manufacture and ripening of Camembert-type cheeses from raw goats' milk. *J App Microbiol*. 85(3), pp.537–544.https://doi.org/10.1046/j.1365-2672.1998.853531.x

Midelet, G., & Carpentier, B. (2002). from Various Materials to Beef. *Society*, *68*(8), 4015–4024. https://doi.org/10.1128/AEM.68.8.4015

Midelet, G., & Carpentier, B. (2006). Construction and Analysis of Fractional Multifactorial Designs To Study Attachment Strength and Transfer of *Listeria monocytogenes* from Pure or Mixed Biofilms after Contact with a Solid Model Food, *72*(4), 2313–2321. https://doi.org/10.1128/AEM.72.4.2313

Miller, A. J., Brown, T., & Call, J. E. (1996). Comparison of Wooden and Polyethylene Cutting Boards : Potential for the Attachment and Removal of Bacteria from Ground

Beef. *Journal of Food Protection*, *59*(8), 854–858. https://doi.org/10.4315/0362-028X-59.8.854

Miller, D. N., & Berry, E. D. (2005). Odor Compounds , Nitrogen Losses , and Dust, *655*, 644–655.

Millet, L., Saubusse, M., Didienne, R., Tessier, L., & Montel, M. C. (2006). Control of *Listeria monocytogenes* in raw-milk cheeses. *International Journal of Food Microbiology*, *108*(1), 105–114. https://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2005.11.004

Montet, M. P., Jamet, E., Ganet, S., Dizin, M., Miszczycha, S., Dunière, L., Thevenot D., & Vernozy-Rozand, C. (2009). Growth and survival of acid-resistant and non-acid-resistant Shiga-toxin-producing *Escherichia coli* strains during the manufacture and ripening of Camembert cheese. *International Journal of Microbiology*, *2009*(1996). https://doi.org/10.1155/2009/653481

Montibus, M., Ismaïl, R., Michel, V., Federighi, M., Aviat, F., & Le Bayon, I. (2016). Assessment of Penicillium expansum and *Escherichia coli* transfer from poplar crates to apples. *Food Control*, *60*, 95–102. https://doi.org/10.1016/j.foodcont.2015.07.025

Moore, G., & Griffith, C. (2007). Problems associated with traditional hygiene swabbing: The need for in-house standardization. *Journal of Applied Microbiology*, *103*(4), 1090–1103. https://doi.org/10.1111/j.1365-2672.2007.03330.x

Moore, G., & Griffith, C. 2002. A comparison of surface sampling methods for detecting coliforms on food contact surfaces. *Food Microbiol,* 19, 65–73.

Moorhead, D. L., Rinkes, Z. L., Sinsabaugh, R. L., & Weintraub, M. N. (2013). Dynamic relationships between microbial biomass, respiration, inorganic nutrients and enzyme activities: Informing enzyme-based decomposition models. *Frontiers in Microbiology*, *4*(AUG), 1–12. https://doi.org/10.3389/fmicb.2013.00223

Moorhead, D. L., Sinsabaugh, R. L., Hill, B. H., & Weintraub, M. N. (2016). Vector analysis of ecoenzyme activities reveal constraints on coupled C, N and P dynamics. *Soil Biology and Biochemistry*, *93*, 1–7. https://doi.org/10.1016/j.soilbio.2015.10.019

Mootian, G., Wu, W.-H., & Matthews, K. R. (2009). Transfer of *Escherichia coli* O157:H7 from Soil, Water, and Manure Contaminated with Low Numbers of the Pathogen to Lettuce Plants. *Journal of Food Protection*, *72*(11), 2308–2312. https://doi.org/10.4315/0362-028X-72.11.2308

Morris, C. E., & Monier, J.-M. (2003). THE ecologlical significance of biofilm formation by plant-associated bacteria. *Annual Review of Phytopathology*, *41*(1), 429–453. https://doi.org/10.1146/annurev.phyto.41.022103.134521

Mourey, A., & Canillac, N. (2002). Anti-*Listeria monocytogenes* activity of essential oils components of conifers. *Food Control*, *13*(4–5), 289–292. https://doi.org/10.1016/S0956-7135(02)00026-9

Moyne, A. L., Sudarshana, M. R., Blessington, T., Koike, S. T., Cahn, M. D., & Harris, L. J. (2011). Fate of *Escherichia coli* O157:H7 in field-inoculated lettuce. *Food Microbiology*, *28*(8), 1417–1425. https://doi.org/10.1016/j.fm.2011.02.001

Muhterem-Uyar, M., Dalmasso, M., Bolocan, A. S., Hernandez, M., Kapetanakou, A. E., Kuchta, T., … Wagner, M. (2015). Environmental sampling for *Listeria monocytogenes* control in food processing facilities reveals three contamination

scenarios. *Food Control*, *51*, 94–107. https://doi.org/10.1016/j.foodcont.2014.10.042
Mukherjee, A., Cho, S., Scheftel, J., Jawahir, S., Smith, K., & Diez-Gonzalez, F. (2006). Soil survival of *Escherichia coli* O157:H7 acquired by a child from garden soil recently fertilized with cattle manure. *Journal of Applied Microbiology*, *101*(2), 429–436. https://doi.org/10.1111/j.1365-2672.2006.02913.x
Muniesa, M., Hammerl, J. A., Hertwig, S., Appel, B., & Br??ssow, H. (2012). Shiga toxin-producing *Escherichia coli* O104:H4: A new challenge for microbiology. *Applied and Environmental Microbiology*, *78*(12), 4065–4073. https://doi.org/10.1128/AEM.00217-12
Murano, E. A., & Pierson, M. D. (1992). Effect of Heat Shock and Growth Atmosphere on the Heat Resistance of *Escherichia coli* O157:H7. *Journal of Food Protection*, *55*(3), 171–175. Retrieved from http://www.ingentaconnect.com/content/iafp/jfp/1992/00000055/00000003/art00005
Muraoka, W., Gay, C., Knowles, D., & Borucki, M. (2003). Prevalence of *Listeria monocytogenes* Subtypes in Bulk Milk of the Pacific Northwest. *Journal of Food Protection*, *66*(8), 1413–1419. https://doi.org/10.4315/0362-028X-66.8.1413
Murinda, S. E., Nguyen, L. T., Ivey, S. J., Gillespie, B. E., Almeida, R. A., Draughon, F. A., & Oliver, S. P. (2002). Molecular Characterization of *Salmonella* spp. Isolated from Bulk Tank Milk and Cull Dairy Cow Fecal Samples. *Journal of Food Protection*, *65*(7), 1100–1105. https://doi.org/10.4315/0362-028X-65.7.1100
Nadon, C. A., Woodward, D. L., Young, C., Rodgers, F. G., & Wiedmann, M. (2001). Correlations between Molecular Subtyping and Serotyping of *Listeria monocytogenes* Correlations between Molecular Subtyping and Serotyping of *Listeria monocytogenes*. *Journal of Clinical Microbiology*, *39*(7), 2704–2707. https://doi.org/10.1128/JCM.39.7.2704
Nataro, J. P. & Kaper J. B. (1998). Diarrheagenic *Escherichia coli*. *Clinical Microbiology Reviews*, *11*(1), 142–201. ; DOI: 10.1128/CMR.11.1.142
Natvig, E. E., Ingham, S. C., Ingham, B. H., Cooperband, L. R., & Roper, T. R. (2002). *Salmonella* enterica serovar typhimurium and *Escherichia coli* contamination of root and leaf vegetables grown in soils with incorporated bovine manure. *Applied and Environmental Microbiology*, *68*(6), 2737–2744. https://doi.org/10.1128/AEM.68.6.2737-2744.2002
Navas, J., Ortiz, S., Lopez, P., Jantzen, M. M., Lopez, V., & Martinez-Suarez, J. V. (2006). Evaluation of effects of primary and secondary enrichment for the detection of *Listeria monocytogenes* by real-time PCR in retail ground chicken meat. *Foodborne Pathogens and Disease*, *3*(4), 347–354. https://doi.org/10.1089/fpd.2006.3.347
Neher, D. A., Weicht, T. R., Bates, S. T., Leff, J. W., & Fierer, N. (2013). Changes in bacterial and fungal communities across compost recipes, preparation methods, and composting times. *PLoS ONE*, *8*(11). http://doi.org/10.1371/journal.pone.0079512
Neher, D. A., Wu, J., Barbercheck, M. E., & Anas, O. (2005). Ecosystem type affects interpretation of soil nematode community measures. *Applied Soil Ecology*, *30*(1), 47–64. http://doi.org/10.1016/j.apsoil.2005.01.002
Nightingale, K. K., Schukken, Y. H., Nightingale, C. R., Fortes, E. D., Ho, A. J., Her, Z., McDonough P.L., Wiedmann, M. (2004). Ecology and Transmission of *Listeria*

monocytogenes Infecting Ruminants and in the Farm Environment, *70*(8), 4458–4467. *Appl Environ Microbiol*. http://doi.org/10.1128/AEM.70.8.4458

Nolan, D. A., Chamblin, D. C., & Troller, J. A. (1992). Minimal water activity levels for growth and survival of *Listeria monocytogenes* and *Listeria innocua*. *International Journal of Food Microbiology*, *16*(4), 323–335. https://doi.org/10.1016/0168-1605(92)90034-Z

Norton, D. M., Mccamey, M. A., Gall, K. L., Scarlett, J. M., Boor, K. J., Wiedmann, M., & Camey, M. A. M. C. (2001). Molecular studies on the ecology of *Listeria monocytogenes* in the smoked fish processing industry. *Applied and Environmental Microbiology*, *67*(1), 198–205. https://doi.org/10.1128/AEM.67.1.198

Norton, D. M., Scarlett, J. M., Horton, K., Sue, D., Thimothe, J., Boor, K. J., & Wiedmann, M. (2001). Characterization and pathogenic potential of *Listeria monocytogenes* isolates from the smoked fish industry. *Applied and Environmental Microbiology*, *67*(2), 646–653. https://doi.org/10.1128/AEM.67.2.646-653.2001

Norwood, D. E., & AGilmour. (2001). The differential adherence capabilities of two *Listeria monocytogenes* strains in monoculture and multispecies biofilms as a function of temperature. *Letters in Applied Microbiology*, *33*(4), 320–324. https://doi.org/10.1046/j.1472-765X.2001.01004.x

Nutrition, C. for F. S. and A. (n.d.). Laboratory Methods - BAM: Detection and Enumeration of *Listeria monocytogenes*.

Nyachuba, D. G., & Donnelly, C. W. (2007). Comparison of 3M™ Petrifilm™ environmental *Listeria* plates against standard enrichment methods for the detection of *Listeria monocytogenes* of epidemiological significance from environmental surfaces. *Journal of Food Science*, *72*(9), 346–354. https://doi.org/10.1111/j.1750-3841.2007.00554.x

Nyarko, E., Kniel, K. E., Zhou, B., Millner, P. D., Luo, Y., Handy, E. T., East C., & Sharma, M. (2017). *Listeria monocytogenes* persistence and transfer to cantaloupes in the packing environment is affected by surface type and cleanliness. *Food Control*. https://doi.org/10.1016/j.foodcont.2017.09.033

O'Brien M., Hunt K., McSweeney S., and Jordan K. (2009). Occurrence of foodborne pathogens in Irish Farmhouse Cheese. *J Food Microbiology*, 26(8), 910–914.

O'Driscoll, B., Gahan, C. G. M., & Hill, C. (1996). Adaptive acid tolerance response in *Listeria monocytogenes*: Isolation of an acid-tolerant mutant which demonstrates increased virulence. *Applied and Environmental Microbiology*, *62*(5), 1693–1698.

Oh, S., Buddenborg, S., Yoder-Himes, D. R., Tiedje, J. M., & Konstantinidis, K. T. (2012). Genomic Diversity of Escherichia Isolates from Diverse Habitats. *PLoS ONE*, *7*(10). https://doi.org/10.1371/journal.pone.0047005

Olaimat, A. N., & Holley, R. A. (2012). Factors influencing the microbial safety of fresh produce: A review. *Food Microbiology*, *32*(1), 1–19. https://doi.org/10.1016/j.fm.2012.04.016

Olde Riekerink, R. G. M., Barkema, H. W., Scholl, D. T., Poole, D. E., & Kelton, D. F. (2010). Management practices associated with the bulk-milk prevalence of *Staphylococcus aureus* in Canadian dairy farms. *Preventive Veterinary Medicine*, *97*(1), 20–28. https://doi.org/https://doi.org/10.1016/j.prevetmed.2010.07.002

Olsen, AR., Gecan, JS., Ziobro, GC., Bryce, JR. (2001). Regulatory Action Criteria for Filth and Other Extraneous Materials V. Strategy for Evaluating Hazardous and

Nonhazardous Filth, Regulatory Toxicology and Pharmacology, 33(3): 363-392. ISSN 0273-2300. https://doi.org/10.1006/rtph.2001.1472.

Olsen, S. J., Patrick, M., Hunter, S. B., Reddy, V., Kornstein, L., MacKenzie, W. R., … Mead, P. (2005). Multistate outbreak of *Listeria monocytogenes* infection linked to delicatessen turkey meat. *Clinical Infectious Diseases : An Official Publication of the Infectious Diseases Society of America*, *40*(7), 962–967. https://doi.org/10.1086/428575

Ortiz, S., López, V., Villatoro, D., López, P., Dávila, J. C., & Martínez-Suárez, J. V. (2010). A 3-Year Surveillance of the Genetic Diversity and Persistence of *Listeria monocytogenes* in an Iberian Pig Slaughterhouse and Processing Plant. *Foodborne Pathogens and Disease*, *7*(10), 1177–1184. https://doi.org/10.1089/fpd.2010.0535

Padhye, N. V, & Doyle, M. P. (1992). *Escherichia coli* 0157 : H7 : Epidemiology , Pathogenesis , and Methods for Detection in Food, *55*(7), 555–565.

Painter, J. A., Hoekstra, R. M., Ayers, T., Tauxe, R. V., Braden, C. R., Angulo, F. J., & Griffin, P. M. (2013). Attribution of foodborne illnesses, hospitalizations, and deaths to food commodities by using outbreak data, United States, 1998-2008. *Emerging Infectious Diseases*, *19*(3), 407–415. https://doi.org/10.3201/eid1903.111866

Pao, S., & Brown, G. E. (1998). Reduction of microorganisms on citrus fruit surfaces during packinghouse processing. *Journal of Food Protection*, *61*(7), 903–906. https://doi.org/10.4315/0362-028X-61.7.903

Parameswaran, S., Guyer, R. B., & Knabel, S. J. (2003). Simple oPSU broth-BAX® PCR-PicoGreen® system for rapid detection of *Listeria monocytogenes* in pasteurized milk and hot dogs. *Journal of Food Safety*, *23*(4), 233–248. https://doi.org/10.1111/j.1745-4565.2003.tb00367.x

Park, H. S., Marth, E. H., Goepfert, J. M., & Olson, N. F. (1972). The fate of *Salmonella* typhimurium in the manufacture and ripening of low-acid Cheddar cheese. *Journal of Milk and Food Technology*, *33*(7), 280–284.

Park, S. H., Chang, P. S., Ryu, S., & Kang, D. H. (2014). Development of a novel selective and differential medium for the isolation of *Listeria monocytogenes*. *Applied and Environmental Microbiology*, *80*(3), 1020–1025. https://doi.org/10.1128/AEM.02840-13

Park, S., Szonyi, B., Gautam, R., Nightingale, K., Anciso, J., & Ivanek, R. (2012). Risk Factors for Microbial Contamination in Fruits and Vegetables at the Preharvest Level: A Systematic Review. *Journal of Food Protection*, *75*(11), 2055–2081. https://doi.org/10.4315/0362-028X.JFP-12-160

Partanen, P., Hultman, J., Paulin, L., Auvinen, P., & Romantschuk, M. (2010). Bacterial diversity at different stages of the composting process. *BMC Microbiology*, *10*, 94. https://doi.org/10.1186/1471-2180-10-94

Paruch, A. M., & Mæhlum, T. (2012). Specific features of *Escherichia coli* that distinguish it from coliform and thermotolerant coliform bacteria and define it as the most accurate indicator of faecal contamination in the environment. *Ecological Indicators*, *23*, 140–142. https://doi.org/10.1016/j.ecolind.2012.03.026

Pascual, C., Robinson, T. P., Ocio, M. J., Aboaba, O. O., & Mackey, B. M. (2008). The effect of inoculum size and sublethal injury on the ability of *Listeria monocytogenes* to initiate growth under suboptimal conditions. *Letters in Applied Microbiology*, *33*(5), 357–361. https://doi.org/10.1046/j.1472-765X.2001.01012.x

Patel, J., Millner, P., Nou, X., & Sharma, M. (2010). Persistence of enterohaemorrhagic and nonpathogenic *E. coli* on spinach leaves and in rhizosphere soil. *Journal of Applied Microbiology*, *108*(5), 1789–1796. https://doi.org/10.1111/j.1365-2672.2009.04583.x

Paul EA (2015) Soil microbiology, ecology, and biochemistry 4th edn. Elsevier, New York.

Pearce, R. B. (1996). Antimicrobial defences in the wood of living trees. *New Phytologist*, *132*(2), 203–233. https://doi.org/10.1111/j.1469-8137.1996.tb01842.x

Peneau, S., Chassaing, D., & Carpentier, B. (2007). First evidence of division and accumulation of viable but nonculturable Pseudomonas fluorescens cells on surfaces subjected to conditions encountered at meat processing premises. *Applied and Environmental Microbiology*, *73*(9), 2839–2846. https://doi.org/10.1128/AEM.02267-06

Pereira, M. L., Carmo, L. S. D. O., Santos, E. J. D. O. S., Pereira, J. L., & Bergdoll, M. S. (1996). Enterotoxin H in Staphylococcal Food Poisoning. *Journal of Food Protection*, *59*(5), 559–561. https://doi.org/10.4315/0362-028X-59.5.559

Pérez-Rodríguez, F., Valero, A., Carrasco, E., García, R. M., & Zurera, G. (2008). Understanding and modelling bacterial transfer to foods: a review. *Trends in Food Science and Technology*, *19*(3), 131–144. https://doi.org/10.1016/j.tifs.2007.08.003

Peters, J. B. (2003). Recommended Methods of Manure Analysis. *Soils.Wisc.Edu*, 1–58. http://doi.org/Recommended Methods of Manure Anaysis (A3769

Petran, R., & Swanson, K. (1993). Simultaneous growth of *Listeria monocytogenes* and *Listeria* innocua. *Journal of Food Protection®*, *56*(7), 616–618. Retrieved from http://www.ingentaconnect.com/content/iafp/jfp/1993/00000056/00000007/art00012

Pintado, C. M. B. S., Oliveira, A., Pampulha, M. E., & Ferreira, M. A. S. S. (2005). Prevalence and characterization of *Listeria monocytogenes* isolated from soft cheese. *Food Microbiology*, *22*(1), 79–85. https://doi.org/10.1016/j.fm.2004.04.004

Pitt, W. M., Harden, T. J., & Hull, R. O. N. R. (2000). Behavior of *Listeria monocytogenes* in Pasteurized Milk during Fermentation with Lactic Acid Bacteria. *Journal of Food Protection*, *63*(7), 916–920. https://doi.org/10.4315/0362-028X-63.7.916

Plumed-Ferrer, C., Väkeväinen, K., Komulainen, H., Rautiainen, M., Smeds, A., Raitanen, J. E., … Von Wright, A. (2013). The antimicrobial effects of wood-associated polyphenols on food pathogens and spoilage organisms. *International Journal of Food Microbiology*, *164*(1), 99–107. https://doi.org/10.1016/j.ijfoodmicro.2013.04.001

Poimenidou, S., Belessi, C. A., Giaouris, E. D., Gounadaki, A. S., Nychas, G. J. E., & Skandamis, P. N. (2009). *Listeria monocytogenes* attachment to and detachment from stainless steel surfaces in a simulated dairy processing environment. *Applied and Environmental Microbiology*, *75*(22), 7182–7188. https://doi.org/10.1128/AEM.01359-09

Poimenidou, S. V., Chrysadakou, M., Tzakoniati, A., Bikouli, V. C., Nychas, G. J., & Skandamis, P. N. (2016). Variability of *Listeria monocytogenes* strains in biofilm formation on stainless steel and polystyrene materials and resistance to peracetic acid and quaternary ammonium compounds. *International Journal of Food Microbiology*, *237*, 164–171. https://doi.org/10.1016/j.ijfoodmicro.2016.08.029

Poimenidou, S. V, Dalmasso, M., Papadimitriou, K., Fox, E. M., Skandamis, P. N., & Jordan, K. (2018). Virulence Gene Sequencing Highlights Similarities and Differences in Sequences in *Listeria monocytogenes* Serotype 1/2a and 4b Strains of Clinical and Food Origin From 3 Different Geographic Locations. *Frontiers in Microbiology*, *9*, 1103. https://doi.org/10.3389/fmicb.2018.01103

Poretsky, R., Rodriguez-R, L. M., Luo, C., Tsementzi, D., & Konstantinidis, K. T. (2014). Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics. *PLoS ONE*, *9*(4). https://doi.org/10.1371/journal.pone.0093827

Pugliese, M., Liu, B. P., Gullino, M. L., & Garibaldi, A. (2008). Selection of antagonists from compost to control soil-borne pathogens. *Journal of Plant Diseases and Protection*, *115*(5), 220–228.

Pradel, N., Bertin, Y., Martin, C., & Livrelli, V. (2008). Molecular analysis of Shiga toxin-producing *Escherichia coli* strains isolated from hemolytic-uremic syndrome patients and dairy samples in France. *Applied and Environmental Microbiology*, *74*(7), 2118–2128. https://doi.org/10.1128/AEM.02688-07

Pritchard, T. J., Beliveau, C. M., Flanders, K. J., & Donnelly, C. W. (1994). Increased Incidence of *Listeria* Species, *57*(September), 770–775.

Pritchard, T. J., & Donnelly, C. W. (1999). Combined secondary enrichment of primary enrichment broths increases *Listeria* detection. *Journal of Food Protection*, *62*(5), 532–535. https://doi.org/10.4315/0362-028X-62.5.532

Public, S. (2013). Outbreak Alert ! 2001 □ 2010 Center for Science in the Public Interest, (March).

Pugliese, M., Liu, B. P., Gullino, M. L., & Garibaldi, A. (2008). Selection of antagonists from compost to control soil-borne pathogens. *Journal of Plant Diseases and Protection*, *115*(5), 220–228.

Rampling, A. (1996). Raw milk cheeses and *Salmonella*. *BMJ : British Medical Journal*, *312*(7023), 67–68. Retrieved from http://www.ncbi.nlm.nih.gov/pmc/articles/PMC2349783/

Ramsaran, H., Chen, J., Brunke, B., Hill, A., & Griffiths, M. W. (1998). Survivial of Bioluminescent *Listeria monocytogenes* and *Escherichia coli* 0157:H7 in Soft Cheeses. *Journal of Dairy Science*, *81*(7), 1810–1817. https://doi.org/10.3168/jds.S0022-0302(98)75750-9

Rangel, J. M., Sparling, P. H., Crowe, C., Griffin, P. M., & Swerdlow, D. L. (2005). Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982-2002. *Emerging Infectious Diseases*, *11*(4), 603–609. https://doi.org/10.3201/eid1104.040739

Rastogi, G., Sbodio, A., Tech, J. J., Suslow, T. V, Coaker, G. L., & Leveau, J. H. J. (2012). Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. *The Isme Journal*, *6*, 1812. Retrieved from http://dx.doi.org/10.1038/ismej.2012.32

Ratnam, S., & March, S. B. (1986). Laboratory studies on *Salmonella*-contaminated cheese involved in a major outbreak of gastroenteritis. *Journal of Applied Bacteriology*, *61*(1), 51–56. https://doi.org/10.1111/j.1365-2672.1986.tb03757.x

Ratnam, S., & March, S. B. (2008). Laboratory studies on *Salmonella*-contaminated cheese involved in a major outbreak of gastroenteritis. *Journal of Applied*

Bacteriology, *61*(1), 51–56. https://doi.org/doi:10.1111/j.1365-2672.1986.tb03757.x
Razzaq, S. (2006). Hemolytic uremic syndrome: an emerging health risk. *American Family Physician*, *74*, 991–996.
Rea, M. C., Cogan, T. M., & Tobin, S. (2018). Incidence of pathogenic bacteria in raw milk in Ireland. *Journal of Applied Bacteriology*, *73*(4), 331–336. https://doi.org/10.1111/j.1365-2672.1992.tb04985.x
Received, S., This, A., Not, W., & Considered, B. E. (2005). Draft Assessment Report Application A499 To Permit The Sale Of Roquefort Cheese Deadline For Public Submissions : 6pm (Canberra time) 4 May 2005, (May), 1–102.
Reissbrodt, R. (2004). New chromogenic plating media for detection and enumeration of pathogenic *Listeria* spp. - An overview. *International Journal of Food Microbiology*, *95*(1), 1–9. https://doi.org/10.1016/j.ijfoodmicro.2004.01.025
Reitsma, C. J., & Henning, D. R. (1996). Survival of enterohemorrhagic *Escherichia coli* O157: H7 during the manufacture and curing of Cheddar cheese. *Journal of Food Protection*, *59*(5), 460–464. Retrieved from http://www.ingentaconnect.com/content/iafp/jfp/1996/00000059/00000005/art00003
Reitzer L (2003) "Nitrogen Assimilation and Global Regulation in *Escherichia coli*" *Annual Review Microbiology* 57: 155-176.
Reynnells, R., Ingram, D. T., Roberts, C., Stonebraker, R., Handy, E. T., Felton, G., Vinyard B.T., Millner P.D. & Sharma, M. (2014). Comparison of U.S. Environmental Protection Agency and U.S. Composting Council Microbial Detection Methods in Finished Compost and Regrowth Potential of *Salmonella* spp. and *Escherichia coli* O157:H7 in Finished Compost. *Foodborne Pathogens and Disease*, *11*(7), 555–567. http://doi.org/10.1089/fpd.2013.1698
Robinson, T. P., Ocio, M. J., Kaloti, A., & Mackey, B. M. (1998). The effect of the growth environment on the lag phase of *Listeria monocytogenes*. *International Journal of Food Microbiology*, *44*(1), 83–92. https://doi.org/https://doi.org/10.1016/S0168-1605(98)00120-2
Rodriguez, A., Autio, W. R., & Mclandsborough, L. A. (2008). Effect of Surface Roughness and Stainless Steel Finish on *Listeria monocytogenes* Attachment and Biofilm Formation. *Journal of Food Protection*, *71*(1), 170–175. https://doi.org/10.4315/0362-028X-71.1.170
Rohrbach, B. W., Draughon, F. A. N. N., Davidson, P. M., & Oliver, S. P. (1992). Prevalence of *Listeria monocytogenes*, Campylobacter jejuni, Yersinia enterocolitica, and *Salmonella* in Bulk Tank Milk: Risk Factors and Risk of Human Exposure. *Journal of Food Protection*, *55*(2), 93–97. https://doi.org/10.4315/0362-028X-55.2.93
Rückerl, I., Muhterem-Uyar, M., Muri-Klinger, S., Wagner, K. H., Wagner, M., & Stessl, B. (2014). *L. monocytogenes* in a cheese processing facility: Learning from contamination scenarios over three years of sampling. *International Journal of Food Microbiology*, *189*, 98–105. https://doi.org/10.1016/j.ijfoodmicro.2014.08.001
Rudolf, M., & Scherer, S. (2001). High incidence of *Listeria monocytogenes* in European red smear cheese. *International Journal of Food Microbiology*, *63*(1), 91–98. https://doi.org/https://doi.org/10.1016/S0168-1605(00)00413-X
Ryser, E.T. 2001. Public Health Concerns, p. 397-546. *In* E. H. Marth and J. L. Steele (eds.), Applied Dairy Microbiology, 2nd ed. Marcel Dekker, Inc. New York, NY.

Ryser, E. T., Arimi, S. M., Marie-Claire Bunduki, M., & Donnelly, C. W. (1996). Recovery of different *Listeria* ribotypes from naturally contaminated, raw refrigerated meat and poultry products with two primary enrichment media. *Applied and Environmental Microbiology*, *62*(5), 1781–1787.

Ryser, E. T., & Marth, E. H. (1987). Behavior of *Listeria monocytogenes* During the Manufacture and Ripening of Cheddar Cheese. *Journal of Food Protection*, *50*(1), 7–13. https://doi.org/10.4315/0362-028X-50.1.7

Sabioni, J. G., Hirooka, E. Y., & de Souza, M. L. (1988). Intoxicação alimentar por queijo minas contaminado com *Staphylococcus aureus*. *Revista de Saude Publica*, *22*(5), 458–461. https://doi.org/10.1590/S0034-89101988000500010

Santorum, P., García, R., López, V., & Martínez-Suárez, J. V. (2012). Review. Dairy farm management and production practices associated with the presence of *Listeria monocytogenes* in raw milk and beef. *Spanish Journal of Agricultural Research*, *10*(2), 360. https://doi.org/10.5424/sjar/2012102-314-11

Sauders, B. D., M. Z. Durak, E. Fortes, K. Windham, Y. Schukken, A. J. Lembo, B. Akey, K. K. Nightingale, & M. Wiedmann. (2006). Molecular characterization of *Listeria monocytogenes* from natural and urban environments. *J. Food Prot.* 69:93–105.

Sauders, B. D., Mangione, K., Vincent, C., Schermerhorn, J., Farchione, C. M., Dumas, N. B., Bopp D., Kornstein L., Fortes E.D., Windham K., & Wiedmann, M. (2004). Distribution of *Listeria monocytogenes* Molecular Subtypes among Human and Food Isolates from New York State Shows Persistence of Human Disease–Associated *Listeria monocytogenes* Strains in Retail Environments. *Journal of Food Protection*, *67*(7), 1417–1428. https://doi.org/10.4315/0362-028X-67.7.1417

Scallan, E., Hoekstra, R. M., Angulo, F. J., Tauxe, R. V., Widdowson, M. A., Roy, S. L., Jones J.L., & Griffin, P. M. (2011). Foodborne illness acquired in the United States-Major pathogens. *Emerging Infectious Diseases*, *17*(1), 7–15. https://doi.org/10.3201/eid1701.P11101

Scharff, R. L. (2012). Economic Burden from Health Losses Due to Foodborne Illness in the United States. *Journal of Food Protection*, *75*(1), 123–131. https://doi.org/10.4315/0362-028X.JFP-11-058

Schlech, W. F. (1983). Epidemic Listeriosis: Evidience for Listeriosis by Food.

Schlech, W. F. (2000). Special Section : Food Safety. *Clinical Infectious Diseases*, *30*(September), 770–775.

Schlesser, J., Gerdes, R., Ravishankar, S., Madsen, K., Mowbray, J., & Teo, A. Y.-L. (2006). Survival of a five-strain cocktail of *Escherichia coli* O157:H7 during the 60-day aging period of cheddar cheese made from unpasteurized milk. *Journal of Dairy Science*, *69*(5), 990–998. https://doi.org/10.4315/0362-028X-69.5.990

Schoder, D., Melzner, D., Schmalwieser, A., Zangana, A., Winter, P., & Wagner, M. (2011). Important Vectors for *Listeria monocytogenes* Transmission at Farm Dairies Manufacturing Fresh Sheep and Goat Cheese from Raw Milk. *Journal of Food Protection*, *74*(6), 919–924. https://doi.org/10.4315/0362-028X.JFP-10-534

Schönwälder, A., Kehr, R., Wulf, A., & Smalla, K. (2002). Wooden boards affecting the survival of bacteria? *Holz Als Roh - Und Werkstoff*, *60*(4), 249–257. https://doi.org/10.1007/s00107-002-0300-6

Schuchat, A., Swaminathan, B., & Broome, C. V. (1991). Epidemiology of human

listeriosis. *Clinical Microbiology Reviews*, *4*(2), 169–183. https://doi.org/10.1128/CMR.4.2.169

Schvartzman, M. S., Maffre, A., Tenenhaus-Aziza, F., Sanaa, M., Butler, F., & Jordan, K. (2011). Modelling the fate of *Listeria monocytogenes* during manufacture and ripening of smeared cheese made with pasteurised or raw milk. *International Journal of Food Microbiology*, *145*, S31–S38. https://doi.org/https://doi.org/10.1016/j.ijfoodmicro.2010.11.032

Semenov, A. V., Van Bruggen, A. H. C., Van Overbeek, L., Termorshuizen, A. J., & Semenov, A. M. (2007). Influence of temperature fluctuations on *Escherichia coli* O157:H7 and *Salmonella* enterica serovar Typhimurium in cow manure. *FEMS Microbiology Ecology*, *60*(3), 419–428. https://doi.org/10.1111/j.1574-6941.2007.00306.x

Shank, F. R., Elliot, L. E., Wachsmuth, L. K., & Losikoff, M. E. (1996). US position on *Listeria monocytogenes* in foods. *Food Control*, *7*(4–5), 253–258. https://doi.org/10.1016/S0956-7135(96)00041-2

Sharma, M., Ingram, D. T., Patel, J. R., Millner, P. D., Wang, X., Hull, A. E., & Donnenberg, M. S. (2009). A novel approach to investigate the uptake and internalization of *Escherichia coli* O157:H7 in spinach cultivated in soil and hydroponic medium. *Journal of Food Protection*, *72*(7), 1513–1520. https://doi.org/10.4315/0362-028X-72.7.1513

Sharma, M., Lakshman, S., Ferguson, S., Ingram, D. T., Luo, Y., & Patel, J. (2011). Effect of Modified Atmosphere Packaging on the Persistence and Expression of Virulence Factors of *Escherichia coli* O157:H7 on Shredded Iceberg Lettuce. *Journal of Food Protection*, *74*(5), 718–726. https://doi.org/10.4315/0362-028X.JFP-10-427

Sharma, M., Millner, P. D., Hashem, F., Camp, M., Whyte, C., Graham, L., & Cotton, C. P. (2016). Survival and Persistence of Nonpathogenic *Escherichia coli* and Attenuated *Escherichia coli* O157:H7 in Soils Amended with Animal Manure in a Greenhouse Environment. *Journal of Food Protection*, *79*(6), 913–921. https://doi.org/10.4315/0362-028X.JFP-15-421

Sharma M. and Reynnells R. (2016). Importance of Soil Amendments: Survival of Bacterial Pathogens in Manure and Compost Used as Organic Fertilizers. *Microbiology Spectrum*, *4*(4), 1–13. http://doi.org/doi:10.1128/microbiolspec.PFS-0010-2015

Shere, J. a, Bartlett, K. J., & Kaspar, C. W. (1998). Longitudinal study of *Escherichia coli* O157 : H7 dissemination on four dairy farms in Wisconsin. *Applied and Environmental Microbiology*, *64*(4), 1390–1399.

Sheth, I., Li, F., Hur, M., Laasri, A., De Jesus, A. J., Kwon, H. J., … Chen, Y. (2018). Comparison of three enrichment schemes for the detection of low levels of desiccation-stressed *Listeria* spp. from select environmental surfaces. *Food Control*, *84*, 493–498. https://doi.org/10.1016/j.foodcont.2017.08.022

Sidhu, B. S., & Beri, V. (1989). Effect of crop residue management on the yields of different crops and on soil properties. *Biological Wastes*, *27*(1), 15–27. https://doi.org/10.1016/0269-7483(89)90027-X

Silk, B. J., Date, K. A., Jackson, K. A., Pouillot, R., Holt, K. G., Graves, L. M., … Mahon, B. E. (2012). Invasive Listeriosis in the Foodborne Diseases Active

Surveillance Network (FoodNet), 2004–2009: Further Targeted Prevention Needed for Higher-Risk Groups. *Clinical Infectious Diseases*, *54*(suppl_5), S396–S404. Retrieved from http://dx.doi.org/10.1093/cid/cis268

Silva, S., Teixeira, P., Oliveira, R., & Azeredo, J. (2008). Adhesion to and viability of *Listeria monocytogenes* on food contact surfaces. *Journal of Food Protection*, *71*(7), 1379–1385. Available at http://www.ncbi.nlm.nih.gov/pubmed/18680936.Accessed on 29 October 2018.

Singh, J. S., Pandey, V. C., & Singh, D. P. (2011). Efficient soil microorganisms: A new dimension for sustainable agriculture and environmental development. *Agriculture, Ecosystems and Environment*, *140*(3–4), 339–353. https://doi.org/10.1016/j.agee.2011.01.017

Singh, R., Jiang, X., & Luo, F. (2010). Thermal inactivation of heat-shocked *Escherichia coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in dairy compost. *Journal of Food Protection*, *73*(9), 1633–1640.

Sinsabaugh, R. L., & Follstad Shah, J. J. (2012). Ecoenzymatic Stoichiometry and Ecological Theory. *Annual Review of Ecology, Evolution, and Systematics*, *43*(1), 313–343. https://doi.org/10.1146/annurev-ecolsys-071112-124414

Sinsabaugh, R. L., Lauber, C. L., Weintraub, M. N., Ahmed, B., Allison, S. D., Crenshaw, C., Contosta A.R., Cusack D., Frey S., Gallo M.E., Gartner T.B., Hobbie S.E., Holland K., Keeler B.L., Powers J.S., Stursova M., Takacs-Vesbach C., Waldrop M.P., Wallenstein M.D., Zak D.R. & Zeglin, L. H. (2008). Stoichiometry of soil enzyme activity at global scale. *Ecology Letters*, *11*(11), 1252–1264. https://doi.org/10.1111/j.1461-0248.2008.01245.x

Sinsabaugh, R. L., & Moorhead, D. L. (1994). Resource allocation to extracellular enzyme production: A model for nitrogen and phosphorus control of litter decomposition. *Soil Biology and Biochemistry*, *26*(10), 1305–1311. https://doi.org/10.1016/0038-0717(94)90211-9

Sivapalasingam, S., Friedman, C. R., Cohen, L., & Tauxe, R. V. (2004). Fresh Produce: A Growing Cause of Outbreaks of Foodborne Illness in the United States, 1973 through 1997. *Journal of Food Protection*, *67*(10), 2342–2353. https://doi.org/10.4315/0362-028X-67.10.2342

Smith J., Buchanan R., Palumbo S., (1983). Effect of food environment on staphylococcal enterotoxin synthesis: A review. *J Food Protection*, 46(6), pp.545–555. Available at: http://www.researchgate.net/publication/257308319_Effect_of_Food_Environment_on_Staphylococcal_Enterotoxin_Synthesis_A_Review/file/60b7d524e1d6ab2cd3.pdf.

Smith-Palmer, A., Stewart, J., & Fyfe, L. (2001). The potential application of plant essential oils as natural food preservatives in soft cheese. *Food Microbiology*, *18*(4), 463–470. https://doi.org/https://doi.org/10.1006/fmic.2001.0415

Spano, G., Goffredo, E., Beneduce, L., Tarantino, D., Dupuy, A., & Massa, S. (2003). Fate of *Escherichia coli* O157:H7 during the manufacture of Mozzarella cheese. *Letters in Applied Microbiology*, *36*(2), 73–76. https://doi.org/10.1046/j.1472-765X.2003.01252.x

Steele, M. L., Mcnab, W. B., Poppe, C., Griffiths, M. W., Chen, S. H. U., Degrandis, S. A., … Odumeru, J. A. (1997). Survey of Ontario Bulk Tank Raw Milk for Food-

Borne Pathogens. *Journal of Food Protection*, *60*(11), 1341–1346. https://doi.org/10.4315/0362-028X-60.11.1341

Strachan, N. J. C., Doyle, M. P., Kasuga, F., Rotariu, O., & Ogden, I. D. (2005). Dose response modelling of *Escherichia coli* O157 incorporating data from foodborne and environmental outbreaks. *International Journal of Food Microbiology*, *103*(1), 35–47. https://doi.org/10.1016/j.ijfoodmicro.2004.11.023

Su, C. (1995). *Escherichia coli* O157: H7 Infection in Humans. *Annals of Internal Medicine*, *123*(9), 698. https://doi.org/10.7326/0003-4819-123-9-199511010-00009

Survival of bacteria on wood and plastic particles: Dependence on wood species and environmental conditions . (2005). *Holzforschung* . https://doi.org/10.1515/HF.2005.012

Swaminathan, B., Barrett, T. J., Hunter, S. B., & Tauxe, R. V. (2001). PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. *Emerging Infectious Diseases*, *7*(3), 382–389. https://doi.org/10.3201/eid0703.017303

Takahashi, H., Kuramoto, S., Miya, S., & Kimura, B. (2011). Desiccation survival of *Listeria monocytogenes* and other potential foodborne pathogens on stainless steel surfaces is affected by different food soils. *Food Control*, *22*(3–4), 633–637. https://doi.org/10.1016/j.foodcont.2010.09.003

Telias, A., White, J. R., Pahl, D. M., Ottesen, A. R., & Walsh, C. S. (2011). Bacterial community diversity and variation in spray water sources and the tomato fruit surface. *BMC Microbiology*, *11*(1), 81. https://doi.org/10.1186/1471-2180-11-81

The Survival of *Listeria monocytogenes* in Aerosols. (1991). *Spurlock*, *71*(4), 750–759.

Those, F. S. A. (2016). Guidance for local enforcement sampling officers on priorities for FSA 2016 / 17 National Coordinated Sampling Programme Priority set A – funded by FSA.

Threlfall, E. J. (2000). Epidemic *Salmonella* typhimurium DT 104—a truly international multiresistant clone. *Journal of Antimicrobial Chemotherapy*, *46*(1), 7–10. Retrieved from http://dx.doi.org/10.1093/jac/46.1.7

Tiedje, J.M., Sexstone, A.J., Parkin, T.B., Revsbech N. P. (1984). *Plant Soil*. 76: 197. https://doi.org/10.1007/BF02205580

Tindall, B. J., Grimont, P. A. D., Garrity, G. M., & Euzéby, J. P. (2005). Nomenclature and taxonomy of the genus *Salmonella*. *International Journal of Systematic and Evolutionary Microbiology*, *55*(1), 521–524. https://doi.org/10.1099/ijs.0.63580-0

Tomás-Callejas, A., López-Velasco, G., Camacho, A. B., Artés, F., Artés-Hernández, F., & Suslow, T. V. (2011). Survival and distribution of *Escherichia coli* on diverse fresh-cut baby leafy greens under preharvest through postharvest conditions. *International Journal of Food Microbiology*, *151*(2), 216–222. https://doi.org/10.1016/j.ijfoodmicro.2011.08.027

Tompkin, R. B. (2002). Control of *Listeria monocytogenes* in the food-processing environment. *Journal of Food Protection*, *65*(4), 709–725. https://doi.org/10.4315/0362-028X-65.4.709

Torres-Vitela, M. R., Mendoza-Bernardo, M., Castro-Rosas, J., Gomez-Aldapa, C. A., Garay-Martinez, L. E., Navarro-Hidalgo, V., & Villarruel-López, A. (2012). Incidence of <I>*Salmonella*,</I> <I>Listeria</I> <I>monocytogenes,</I> <I>Escherichia</I>

<I>coli</I> O157:H7, and Staphylococcal Enterotoxin in Two Types of Mexican Fresh Cheeses. *Journal of Food Protection*, *75*(1), 79–84. https://doi.org/10.4315/0362-028X.JFP-11-258

Trmčić, A., Chauhan, K., Kent, D. J., Ralyea, R. D., Martin, N. H., Boor, K. J., & Wiedmann, M. (2016). Coliform detection in cheese is associated with specific cheese characteristics, but no association was found with pathogen detection. *Journal of Dairy Science*, *99*(8), 6105–6120. https://doi.org/10.3168/jds.2016-11112

Trmčić, A., Ralyea, R., Meunier-Goddik, L., Donnelly, C., Glass, K., D'Amico, D., Meredith E., Kehler M., Tranchina N., McCue C., & Wiedmann, M. (2017). Consensus categorization of cheese based on water activity and pH—A rational approach to systemizing cheese diversity. *Journal of Dairy Science*, *100*(1), 841–847. http://doi.org/10.3168/jds.2016-11621

Turner, S. M., Scott-Tucker, A., Cooper, L. M., & Henderson, I. R. (2006). Weapons of mass destruction: Virulence factors of the global killer enterotoxigenic *Escherichia coli*. *FEMS Microbiology Letters*, *263*(1), 10–20. https://doi.org/10.1111/j.1574-6968.2006.00401.x

United States Department of Agriculture (USDA). (2014). Guidelines for Organic Crop Certification.

United States Department of Agriculture, Food Safety Inspection Services (USDA/FSIS). (2012). FSIS Compliance Guideline : Controlling *Listeria monocytogenes* in Post-lethality Exposed Ready-to-Eat Meat and Poultry Products, (January), 2–43. Retrieved from http://www.fsis.usda.gov/wps/wcm/connect/d3373299-50e6-47d6-a577-e74a1e549fde/Controlling_LM_RTE_Guideline_0912?MOD=AJPERES. Accessed 29 October 2018.

United States Department of Agriculture, Food Safety Inspection Services (USDA/FSIS). (2003). Control of *Listeria monocytogenes* in Ready-to-Eat Meat and Poultry Products; Final Rule. https://doi.org/10.2307/1166652

United States Department of Agriculture, Food Safety Inspection Services (USDA/FSIS). (1999). USA Delegation Report for the Codex committee on Food Hygiene drafting Group Meeting for the Code of Hygienic Practice for Milk and Milk Products," 22-25- March 1999. P. 2. Available from www.fsis.usda.gov:80/OA/codex/rep_brussesl.htm.

U.S. Food and Drug Administration (FDA). (n.d.). FDA Fact Sheet: Produce Safety Rule. Retrieved from http://www.fda.gov/ForConsumers/ConsumerUpdates/ucm107358.htm

U.S. Food and Drug Administration (FDA). (2018). CPG Sec. 555.320 *Listeria monocytogenes*.

U.S. Food and Drug Administration (FDA). (2018). CFR Title 21 Sec. 1240.61 Mandatory pasteurization for all milk and milk products in final package form intended for direct human consumption. https://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfcfr/CFRSearch.cfm?fr=1240.61. Accessed December 3, 2018.

U.S. Food and Drug Administration (FDA). (2018). FSMA Final Rule on Produce Safety. https://www.fda.gov/food/guidanceregulation/fsma/ucm334114.htm. Accessed Nov 4, 2018.

U.S Food and Drug Administration (FDA). (2017).Bacterialogical Analytical Manual

(BAM): Detection and Enumeration of *Listeria monocytogenes* Chapter 10. https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm071400.htm. Accessed on 29 October 2018.

U.S. Food and Drug Administration (FDA). (2017) (revised from 1995). Bacteriological Analytical Manual (BAM): Diarrheagenic *Escherichia coli*. Bacteriological Analytical Manual. Chapter 4A. https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm070080.htm. Accessed 9.29.2017.

U.S. Food and Drug Administration (FDA). (2016). Center for Food Safety and Applied Nutrition (CFSAN). FY 2014 – 2016 Microbiological Sampling Assignment Summary report: raw milk cheese aged 60 days. Available at: http://www.fda.gov/Food/NewsEvents/ConstituentUpdates/default.htm?source=govdelivery&utm_medium=email&utm_source=govdelivery. Accessed 7.26.2016.

U.S. Food and Drug Administration (FDA) Center for Food Safety and Applied Nutrition CFSAN) (2014). CFSAN Constituent Update. Clarification on Using Wood Shelving in Artisanal Cheesemaking. http://wayback.archive-it.org/7993/20171114120232/https:/www.fda.gov/Food/NewsEvents/ConstituentUpdates/ucm400808.htm. Accessed December 3, 2018.

U.S Food and Drug Administration (FDA). (2016). FSMA Rules and Guidance for Industry. http://www.fda.gov/Food/GuidanceRegulation/FSMA/ucm334552.htm. Accessed March 20, 2016.

U.S. Food and Drug Administration (FDA). (2016). FDA Investigated Multistate Outbreak of *Listeria* in Dole Leafy Greens Products Produced in the Dole Facility in Springfield, Ohio. https://www.fda.gov/food/recallsoutbreaksemergencies/outbreaks/ucm482807.htm. Accessed November 4, 2018.

U.S. Food and Drug Administration (FDA). (2015). Food Safety modernization Act (FSMA). U.S. Food and Drug Administration, Washington, DC. Accessed November 4, 2018. http://www.fda.gov/Food/GuidanceRegulation/FSMA

U.S. Food and Drug Administration (FDA). (2015) FSMA Final Rule for Preventive Controls for Human Food. U.S. Food and Drug Administration, Silver Spring, MD. Accessed November 4, 2018. http://www.fda.gov/Food/GuidanceRegulation/FSMA/ucm334115.htm

U.S. Food and Drug Administration FDA. enter for Food Safety and Applied Nutrition (USFDA/CFSAN) (2015). Testing Methodology for *Listeria* species or *L. monocytogenes* in Environmental Samples, (October), 1–11.

U.S. Food and Drug Administration (FDA). (2014). FDA at a Glance Key Revisions on Produce Safety, 1–2.

U.S. Food and Drug Administration (FDA). (2014). FDA Update on the Status of Artisanal Cheese. http://www.fda.gov/Food/NewsEvents/ucm413309.htm. Accessed 8.26.2015.

U.S. Food and Drug Administration. (2012). Bad bug book: Handbook of Foodborne Pathogenic Microorganisms and Natural Toxins. *Bad Bug Book: Handbook of Foodborne Pathogenic Microorganisms and Natural Toxins*, 292. https://doi.org/10.1016/S1872-2040(10)60451-3

U.S. Food and Drug Administration (FDA). (2013). FDA's International Food Safety Capacity Building Plan. Food Safety Modernization Act Section 305. http://www.fda.gov/downloads/Food/GuidanceRegulation/UCM341440.pdf. Accessed 11.29.16.

U.S. Food and Drug Administration (FDA). (2013). Standards for the Growing, Harvesting, Packing, and Holding of Produce for Human Consumption; Proposed Rule. *2013*, 1–548.

U.S. Food and Drug Administration (FDA). (2011). FSMA Facts: background on the FDA food safety modernization act (FMSA). *FDA Food Safety Modernization Act*, 1–3. Retrieved from http://www.fda.gov/Food/GuidanceRegulation/FSMA/ucm239907.htm Accessed November 4, 2018.

U.S. Food and Drug Administration (FDA). (2010). Bacteriological Analytical Manual (BAM) Appendix 2: Most Probable Number from Serial Dilutions. https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm109656.htm. Accessed 4.13.17.

U.S. Food and Drug Administration (FDA). (2002). Bacteriological Analytical Manual (BAM): Enumeration of *Escherichia coli* and the Coliform Bacteria. Bacteriological Analytical Manual. Chapter 4. http://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm064948.htm Accessed 6.22.2015.

U.S. Food and Drug Administration (FDA). (1995). Bacteriological Analytical Manual (BAM): Diarrheagenic *Escherichia coli*. Bacteriological Analytical Manual. Chapter 4A; 8th Edition.

U.S Food and Drug Administration (FDA). Center for Food Safety and Applied Nutrition (USFDA/CFSAN). (2018). FSMA final rule for preventive controls for human food. Available at https://www.fda.gov/food/guidanceregulation/fsma/ucm334115.htm. Accessed on 29 October 2018.

U.S Food and Driug Administration (FDA). Center for Food Safety and Applied Nutrition. (USFDA/CFSAN). (2017). Control of *Listeria monocytogenes* in ready-to-eat foods: guidance for industry draft guidance. *International Journal of Pharmaceutical & Biological Archives*, *3*(Draft Guidance), 1–49. Available at https://www.fda.gov/downloads/food/guidanceregulation/guidancedocumentsregulatoryinformation/ucm535981.pdf. Accessed on 29 October 2018.

U.S Food and Drug Administration (FDA) Center for Food Safety and Applied Nutrition (USFDA/CFSAN) (2015). Testing Methodology for *Listeria* species or L . monocytogenes in Environmental Samples, (October), 1–11.

U.S. Food and Drug Administration (FDA). Center for Food Safety and Applied Nutrition (CFSAN) (2010). Compliance Policy Guide Sec. 527.300 Dairy Products-Microbial Contaminants and Alkaline Phosphatase Activity. 75 FR 80826. http://origin.www.gpo.gov/fdsys/pkg/FR-2010-12-12/pdf/2010-32232.pdf. Accessed 11.17.14.

U.S. Food and Drug Administration (FDA). (2009). Center for Food Safety and Applied Nutrition (CFSAN). Draft Compliance Policy Guide Sec. 527.300 Dairy Products-Microbial Contaminants and Alkaline Phosphatase Activity. 74 FR 62795. http://edocket.access.gpo.gov/2009/pdf/E9-28756.pdf. Accessed 11.9.14.

www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?CFRPart=112
U.S Food and Drug Administration. Department of Health and Human Services (USFDA/HHS). (2018). Code of Federal Regulation Title 21. Current Good Manufacturing Practice in Maufacturing, Packing, or Holding Human Food. www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?CFRPart=112. Accessed November 3, 2018.
U.S. Food and Drug Administration, Department of Health & Human Services (USFDA/HHS) (2017). FDA Form 483 "Vulto creamery inspectional observation report"
U.S. Food and Drug Administration (FDA) Department of Health and Human Services (HHS) (2014). FDA's Response Letter to the American Cheese Society. http://www.cheesesociety.org/wp-content/uploads/2014/11/Response-to-American-Cheese-Society.pdf?utm_source=Copy+of+Member+Update-CPG+for+Non-Toxigenic+E.+coli&utm_campaign=Member+Update+11-5-14&utm_medium=email. Accessed 8.2.2015.
U.S. Food and Drug Administration, Department of Health & Human Services (USFDA/HHS). (2013). Part III Department of Health and Human Services. *Federal Register*, *80*(200), 1–195. Retrieved from http://webapps.dol.gov/federalregister/PdfDisplay.aspx?DocId=26927
U.S. Food and Drug Administration, Health & Human Services (FDA/HHS). (2013) Report to Congress: Ensuring a Safe Food Supply. https://www.fda.gov/downloads/food/guidanceregulation/fsma/ucm351876.pdf. Accessed November 4, 2018.
U.S. Food and Drug Administration, Department of Health & Human Services (FDA/HHS). (2013). Part II Department of Health and Human Services, *78*(11), 1–144.
U.S. Food and Drug Administration (FDA), & Health Canada. (2015). Joint FDA / Health Canada Quantitative Assessment of the Risk of Listeriosis from Soft-Ripened Cheese Consumption in the United States and Canada : Draft Report ., (July), 9.
U.S. Food and Drug Administration, Office of Regulatory Affairs (USFDA/ORA) 2005. Sec. 527.300 Pathogens in Dairy Products (CPG 7106.08). Available at: http://www.fda.gov/ora/compliance_ref/cpg/cpgfod/cpg527-300.html. Accessed 20 May 2008.
Uhlich, G. A., Luchansky, J. B., Tamplin, M. L., Molina-Corral, F. J., Anandan, S., & Porto-Fett, A. C. S. (2006). Effect of storage temperature on the growth of *Listeria monocytogenes* on Queso Blanco slices. *Journal of Food Safety*, *26*(3), 202–214. https://doi.org/10.1111/j.1745-4565.2006.00043.x
Ukuku, D. O., & Fett, W. (2002). Behavior of *Listeria monocytogenes* inoculated on cantaloupe surfaces and efficacy of washing treatments to reduce transfer from rind to fresh-cut pieces. *Journal of Food Protection*, *65*(6), 924–930. https://doi.org/10.4315/0362-028X-65.6.924
Valero, A., Pérez-rodríguez, F., Carrasco, E., Fuentes-alventosa, J. M., García-gimeno, R. M., & Zurera, G. (2009). International Journal of Food Microbiology Modelling the growth boundaries of *Staphylococcus aureus* : Effect of temperature , pH and water activity. *International Journal of Food Microbiology*, *133*(1–2), 186–194. https://doi.org/10.1016/j.ijfoodmicro.2009.05.023

Välimaa, A. L., Honkalampi-Hämäläinen, U., Pietarinen, S., Willför, S., Holmbom, B., & von Wright, A. (2007). Antimicrobial and cytotoxic knotwood extracts and related pure compounds and their effects on food-associated microorganisms. *International Journal of Food Microbiology*, *115*(2), 235–243. https://doi.org/10.1016/j.ijfoodmicro.2006.10.031

Van den Brink, P. J., Van den Brink, N. W., & Ter Braak, C. J. F. (2003). Multivariate Analysis of Ecotoxicological Data Using Ordination: Demonstrations of Utility on the Basis of Various Examples. *Australasian Journal of Ecotoxicology*, *9*, 141–156. Retrieved from http://www.biometris.wur.nl/NR/rdonlyres/71EBBDE7-DE7B-4956-9BEA-2B807C34A8EF/47864/terBraak2003VandenBrink2Braak_AJE.pdf

Van Den Brink, P. J., & Ter Braak, C. J. F. (1999). Principal response curves: Analysis of time-dependent multivariate responses of biological community to stress. *Environmental Toxicology and Chemistry*, *18*(2), 138–148. http://doi.org/10.1897/1551-5028(1999)018<0138:PRCAOT>2.3.CO;

Van Elsas, J. D., Semenov, A. V., Costa, R., & Trevors, J. T. (2011). Survival of *Escherichia coli* in the environment: Fundamental and public health aspects. *ISME Journal*, *5*(2), 173–183. https://doi.org/10.1038/ismej.2010.80

Van Kessel, J. S., Karns, J. S., Gorski, L., McCluskey, B. J., & Perdue, M. L. (2004). Prevalence of *Salmonella*e, *Listeria monocytogenes*, and Fecal Coliforms in Bulk Tank Milk on US Dairies*. *Journal of Dairy Science*, *87*(9), 2822–2830. https://doi.org/https://doi.org/10.3168/jds.S0022-0302(04)73410-4

Van Stelten, A., Simpson, J. M., Ward, T. J., & Nightingale, K. K. (2010). Revelation by single-nucleotide polymorphism genotyping that mutations leading to a premature stop codon in inlA are common among *Listeria monocytogenes* isolates from ready-to-eat foods but not human listeriosis cases. *Applied and Environmental Microbiology*, *76*(9), 2783–2790. https://doi.org/10.1128/AEM.02651-09

Verhaegen, B., De Reu, K., Heyndrickx, M., & De Zutter, L. (2015). Comparison of Six Chromogenic Agar Media for the Isolation of a Broad Variety of Non-O157 Shigatoxin-Producing *Escherichia coli* (STEC) Serogroups. *International Journal of Environmental Research and Public Health* . https://doi.org/10.3390/ijerph120606965

Vernozy-Rozand, C., Meyrand, A., Mazuy, C., Delignette-Muller, M. L., Jaubert, G., Perrin, G., … Richard, Y. (1998). Behaviour and enterotoxin production by *Staphylococcus aureus* during the manufacture and ripening of raw goats' milk lactic cheeses. *Journal of Dairy Research*, *65*(2), 273–281. https://doi.org/10.1017/S0022029997002781

Vidovic, S., Mangalappalli-Illathu, A. K., & Korber, D. R. (2011). Prolonged cold stress response of *Escherichia coli* O157 and the role of rpoS. *International Journal of Food Microbiology*, *146*(2), 163–169. https://doi.org/10.1016/j.ijfoodmicro.2011.02.018

Vivegnis, J., El-Lioui, M., Leclercq, A., Lambert, B., & Decallonne, J. (1999). Detection of Shiga-like toxin producing *Escherichia coli* from raw milk cheeses produced in Wallonia. *BASE: Biotechnologie, Agronomie, Societe et Environnement*, *3*(3), 159–164.

Voetsch, A. C., Angulo, F. J., Jones, T. F., Moore, M. R., Nadon, C., McCarthy, P., Shiferaw B., Megginson M.B., Hurd S., Anderson B.J., Cronquist A., Vugia D.J.,

Medus C., Segler S., Graves L.M., Hoekstra R.M. & Griffin, P. M. (2007). Reduction in the incidence of invasive listeriosis in foodborne diseases active surveillance network sites, 1996-2003. *Clinical Infectious Diseases*, *44*(January), 513–520. https://doi.org/10.1086/511006

Vorst, K. L., Todd, E. C. D., & Ryser, E. T. (2004). Improved quantitative recovery of *Listeria monocytogenes* from stainless steel surfaces using a one-ply composite tissue. *Journal of Food Protection*, *67*(10), 2212–2217. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/15508632

Wagner, M., & Allerberger, F. (2003). Characterization of *Listeria monocytogenes* recovered from 41 cases of sporadic listeriosis in Austria by serotyping and pulsed-field gel electrophoresis. *FEMS Immunology and Medical Microbiology*, *35*(3), 227–234. https://doi.org/10.1016/S0928-8244(02)00445-5

Wagner, M., Maderner, A., & Brandl, E. (1996). Random amplification of polymorphic DNA for tracing and molecular epidemiology of *Listeria* contamination in a cheese plant. *Journal of Food Protection*, *59*(4), 384–389. https://doi.org/10.4315/0362-028X-59.4.384

Walk, S. T., Alm, E. W., Gordon, D. M., Ram, J. L., Toranzos, G. A., Tiedje, J. M., & Whittam, T. S. (2009). Cryptic lineages of the genus Escherichia. *Applied and Environmental Microbiology*, *75*(20), 6534–6544. https://doi.org/10.1128/AEM.01262-09

Wallace, J. S., Cheasty, T., & Jones, K. (1997). Isolation of Vero cytotoxin-producing *Escherichia coli* O157 from wild birds. *Journal of Applied Microbiology*, *82*(3), 399–404. https://doi.org/10.1046/j.1365-2672.1997.00378.x

Walsh, D., Duffy, G., & Sheridan, J. J. (1998). Comparison of Selective and Nonselective Enrichment Media for the Isolation of Listerza Species From Retail Foods, *18*, 85–99.

Wang Q, Garrity GM, Tiedje JM, Cole JR (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl Environ Microbiol 73:5261–5267

Weller, D., Andrus, A., Wiedmann, M., & den Bakker, H. C. (2015). *Listeria* booriae sp. nov. and *Listeria* newyorkensis sp. nov., from food processing environments in the USA. *International Journal of Systematic and Evolutionary Microbiology*, *65*(1), 286–292. Retrieved from http://ijs.microbiologyresearch.org/content/journal/ijsem/10.1099/ijs.0.070839-0

Weller, D. L., Kovac, J., Roof, S., Kent, D. J. D., Tokman, J. I., Kowalcyk, B., … Wiedmann, M. (2017). Survival of *Escherichia coli* on lettuce under field conditions encountered in the Northeastern United States. *Journal of Food Protection*, *80*(7), 1214–1221. https://doi.org/https://doi.org/10.4315/0362-028X.JFP-16-419

Wells, J., & Shipman, L. (1991). Isolation of *Escherichia coli* Serotype 0157 : H7 and Other Shiga-Like-Toxin-Producing E . coli from Dairy Cattle. *Journal of Clinical Microbiology*, *29*(5), 985–989. https://doi.org/0095-1137/91/050985-05$02.00/0

Wemmenhove, E., Beumer, R. R., van Hooijdonk, A. C. M., Zwietering, M. H., & Wells-Bennik, M. H. J. (2014). The fate of *Listeria monocytogenes* in brine and on Gouda cheese following artificial contamination during brining. *International Dairy Journal*, *39*(2), 253–258. https://doi.org/https://doi.org/10.1016/j.idairyj.2014.06.002

Westerholt, S., Pieper, A.-K., Griebel, M., Volk, H.-D., Hartung, T., & Oberhoffer, R. (2003). Characterization of the Cytokine Immune Response in Children Who Have Experienced an Episode of Typical Hemolytic-Uremic Syndrome. *Clin. Diagn. Lab. Immunol.*, *10*(6), 1090–1095. https://doi.org/10.1128/CDLI.10.6.1090-1095.2003

Whalley, W. R., Ober, E. S., & Jenkins, M. (2013). Measurement of the matric potential of soil water in the rhizosphere. *Journal of Experimental Botany*, *64*(13), 3951–3963. http://doi.org/10.1093/jxb/ert044

Whitmore. (2012). Habitat Ordination of Passerine Birds of the Virgin River Valley , Southwestern Utah Author (s): Robert C . Whitmore Reviewed work (s): Source : The Wilson Bulletin , Vol . 87 , No . 1 (Mar ., 1975), pp . 65-74 Published by : Wilson Ornithological S, *87*(1), 65–74.

Wiedmann, M. (2002). Molecular subtyping methods for *Listeria monocytogenes*. *Journal of AOAC International*, *85*(2), 524–531.

Wiedmann, M., Bruce, J. L., Keating, C., Johnson, A. E., McDonough, P. L., & Batt, C. A. (1997). Ribotypes and virulence gene polymorphisms suggest three distinct *Listeria monocytogenes* lineages with differences in pathogenic potential. *Infection and Immunity*, *65*(7), 2707–2716. Retrieved from http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=175382&tool=pmcentrez&rendertype=abstract

Williams, T. R., Moyne, A. L., Harris, L. J., & Marco, M. L. (2013). Season, Irrigation, Leaf Age, and *Escherichia coli* Inoculation Influence the Bacterial Diversity in the Lettuce Phyllosphere. *PLoS ONE*, *8*(7), 1–14. https://doi.org/10.1371/journal.pone.0068642

Wood, J. D., Bezanson, G. S., Gordon, R. J., & Jamieson, R. (2010). Population dynamics of *Escherichia coli* inoculated by irrigation into the phyllosphere of spinach grown under commercial production conditions. *International Journal of Food Microbiology*, *143*(3), 198–204. https://doi.org/10.1016/j.ijfoodmicro.2010.08.022

World Bioproducts (n.d.). The Polyurethane Advantage. Available at http://www.worldbioproducts.com/polyurethane.html. Accessed on 20 October 2018.

World Trade Organization (WTO). (1998). General Agreement on Tariffs and Trade. https://www.wto.org/english/res_e/booksp_e/agrmntseries2_gatt_e.pdf. Accessed 4.15.17

Wu, J., S. C. Long, D. Das, and S. M. Dorner. (2011). Are microbial indicators and pathogens correlated? A statistical analysis of 40 years of research. J. Water Health 9:265–278. http://dx.doi.org/10.2166/wh.2011.117

Wulff, G., Gram, L., Ahrens, P., & Vogel, B. F. (2006). One group of genetically similar *Listeria monocytogenes* strains frequently dominates and persists in several fish slaughter- and smokehouses. *Applied and Environmental Microbiology*, *72*(6), 4313–4322. https://doi.org/10.1128/AEM.02288-05

Yang, C.-H., Crowley, D. E., Borneman, J., & Keen, N. T. (2001). Microbial phyllosphere populations are more complex than previously realized. *Proceedings of the National Academy of Sciences*, *98*(7), 3889–3894. https://doi.org/10.1073/pnas.051633898

Yao, Z., Yang, L., Wang, H., Wu, J., & Xu, J. (2015). Fate of *Escherichia coli* O157: H7

in agricultural soils amended with different organic fertilizers. *Journal of Hazardous Materials*, *296*, 30–36. https://doi.org/10.1016/j.jhazmat.2015.04.023

Yeoh YK, Sekiguchi Y, Parks DH, and Hugenholtz P.. Comparative Genomics of Candidate Phylum TM6 Suggests That Parasitism Is Widespread and Ancestral in This Lineage. Mol. Biol.Evol.33(4):915–927

Yoshitomi, K. J., Jinneman, K. C., & Weagant, S. D. (2003). Optimization of a 3′-minor groove binder-DNA probe targeting the uidA gene for rapid identification of *Escherichia coli* O157:H7 using real-time PCR. *Molecular and Cellular Probes*, *17*(6), 275–280. https://doi.org/10.1016/j.mcp.2003.07.001

Yousef, A. E., & Marth, E. H. (1990). Fate of *Listeria monocytogenes* During the Manufacture and Ripening of Parmesan Cheese. *Journal of Dairy Science*, *73*(12), 3351–3356. https://doi.org/https://doi.org/10.3168/jds.S0022-0302(90)79030-3

Yousef, A. E., & Marth, E. H. (1988). Behavior of *Listeria monocytogenes* During the Manufacture and Storage of Colby Cheese. *Journal of Food Protection*, *51*(1), 12–15. https://doi.org/10.4315/0362-028X-51.1.12

Zaleski, K. J., Josephson, K. L., Gerba, C. P., & Pepper, I. L. (2005). Potential Regrowth and Recolonization of *Salmonella*e and Indicators in Biosolids and Biosolid-Amended Soil -- Zaleski et al. 71 (7): 3701 -- Applied and Environmental Microbiology, *71*(7), 3701–3708. https://doi.org/10.1128/AEM.71.7.3701

Zangerl, P., Matlschweiger, C., Dillinger, K., & Eliskases-Lechner, F. (2010). Survival of *Listeria monocytogenes* after cleaning and sanitation of wooden shelves used for cheese ripening. *European Journal of Wood and Wood Products*, *68*(4), 415–419. https://doi.org/10.1007/s00107-009-0381-6

Zárate, V., Belda, F., Pérez, C., & Cardell, E. (1997). Changes in the microbial flora of Tenerife goats' milk cheese during ripening. *International Dairy Journal*, *7*(10), 635–641. https://doi.org/https://doi.org/10.1016/S0958-6946(97)00065-4

Zehren, V. L., & Zehren, V. F. (1968a). Examination of Large Quantities of Cheese for Staphylococcal Enterotoxin A. *Journal of Dairy Science*, *51*(5), 635–644. https://doi.org/10.3168/jds.S0022-0302(68)87047-X

Zehren, V. L., & Zehren, V. F. (1968b). Relation of Acid Development During Cheesemaking to Development of Staphylococcal Enterotoxin A. *Journal of Dairy Science*, *51*(5), 645–649. https://doi.org/10.3168/jds.S0022-0302(68)87048-1

Zhang, X., McDaniel, A. D., Wolf, L. E., Keusch, G. T., Waldor, M. K., & Acheson, D. W. K. (2000). Quinolone Antibiotics Induce Shiga Toxin–Encoding Bacteriophages, Toxin Production, and Death in Mice. *The Journal of Infectious Diseases*, *181*(2), 664–670. https://doi.org/10.1086/315239

Zhang, Y., Laing, C., Steele, M., Ziebell, K., Johnson, R., Benson, A. K., Taboada, E., & Gannon, V. P. J. (2007). Genome evolution in major *Escherichia coli* O157:H7 lineages. *BMC Genomics*, *8*. https://doi.org/10.1186/1471-2164-8-121

Zhao, T., Doyle, M. P., & Zhao, P. (2004). Control of *Listeria monocytogenes* in a Biofilm by Competitive-Exclusion Microorganisms. *Society*, *70*(7), 3996–4003. https://doi.org/10.1128/AEM.70.7.3996

Zitz, U., Zunabovic, M., Domig, K. J., Wilrich, P.-T., & Kneifel, W. (2011). Reduced Detectability of *Listeria monocytogenes* in the Presence of *Listeria* innocua. *Journal of Food Protection*, *74*(8), 1282–1287. https://doi.org/10.4315/0362-028X.JFP-11-045

Zweifel, C., Giezendanner, N., Corti, S., Krause, G., Beutin, L., Danuser, J., & Stephan, R. (2010). Characteristics of Shiga Toxin–Producing *Escherichia coli* Isolated from Swiss Raw Milk Cheese within a 3-Year Monitoring Program. *Journal of Food Protection*, *73*(1), 88–91. https://doi.org/10.4315/0362-028X-73.1.88