2019

Comparison of Methods for Detection of Listeria on Wooden Shelves used for Cheese Aging: Challenges Associated with Sampling Porous Surfaces

Gina Christine Frontino
University of Vermont

COMPARISON OF METHODS FOR DETECTION OF *LISTERIA* ON WOODEN SHELVES USED FOR CHEESE AGING: CHALLENGES ASSOCIATED WITH SAMPLING POROUS SURFACES

A Thesis Presented

by

Gina Frontino

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
for the Degree of Master of Science
Specializing in Nutrition and Food Science

January, 2019

Defense Date:11/9/2018
Thesis Examination Committee

Catherine Donnelly, Ph.D. Advisor
Douglas Johnson, Ph.D., Chairperson
Omar Oyarzabal, Ph.D.
Cynthia J. Forehand, Ph.D., Dean of the Graduate College

ABSTRACT

This thesis examined the efficacy of various sampling and detection methods used for environmental monitoring of *Listeria* species on wooden surfaces used for cheese aging. Government agencies including the Food and Drug Administration (FDA) and United States Department of Agriculture (USDA) recommend enrichment methods coupled with use of environmental sponges and swabs. Our study compared efficacy of sponge swabs manufactured by 3M™ and World Bioproducts. There is a lack of research validating the best performing swab type and enrichment method combination that is sensitive when used on rough, porous surfaces. The sensitivity of these environmental sampling tools and methods are critical considerations to effectively monitor the presence of *Listeria* species on wooden boards used during aging of artisan cheese.

Seasoned spruce wooden shelves, cut into 100cm^2 replicates, were spot inoculated with varying concentrations of *Listeria* species inocula, the *Listeria* species strains consisted of two *L. monocytogenes* strains and a Green Florescence Protein (GFP) expressing strain of *L. innocua.* The inoculated wooden surface was swabbed with three environmental sampling sponge/swab formats (World Bioproducts© EZ Reach™ environmental swabs (WBEZ) with HiCap (WBHC) and Dey-Engley (WBDE) neutralizing broths; and 3M™ environmental swabs (3M™) with Dey-Engley neutralizing broth). Enumeration methods were used to determine the low target limits of detection. Once the low target concentrations were identified, five enrichment methods consisting of 3M™ *Listeria* Environmental Plate, FDA, Dual Enrichment, modified USDA, and modified FDA were challenged against low concentrations of *Listeria* species inocula (0.01 cfu/cm^2, 0.1 cfu/cm^2, 1 cfu/cm^2) and the three environmental sponge swab formats. Performance of the swab formats was assessed by collection of naturally contaminated environmental samples (n=405) from dairy farm environments, swabbing where wooden surfaces existed, and analyzed using the most effective enrichment methods found from previous experiments. Lastly, the wooden surfaces and sponge swabs were observed under a Florescent Microscope using GFP *L. innocua* to visually determine how each sponge material of the 3M™ and World Bioproducts recovered the inocula.

When wood surfaces were inoculated at high concentration levels of *Listeria* spp., all swab formats performed equally for detecting *Listeria*. Success of positive recovery at low concentrations was variable, where enrichment methods and swabs were not dependent on each other. The swab format that worked best for detecting low levels of *Listeria* species was the WBDE sponge swab. The WBDE swab also performed the best in dairy farm environmental sampling. The m-USDA enrichment method was found to be most effective in recovery and repair of low and potentially injured *Listeria* spp. Wooden surfaces are rough and porous and should be taken into consideration when creating an environmental sampling plan for these food contact surfaces. All swabs and methods performed with only slight variation, but the variation could be significant when monitoring wooden shelves with low level contamination of *Listeria* species. Artisan cheesemakers who use wooden shelves during the aging of their cheese, should ensure use of the most sensitive detection method

ACKNOWLEDGEMENTS

It has been a privilege to be given the opportunity to pursue a graduate research assistantship at the University of Vermont for a Master of Science. During my time as a graduate student, I have reformed my scientific laboratory skills, and gained intellectual independence. I would like to express my sincerest gratitude to my advisor Dr. Catherine Donnelly for her expertise and motivation as I moved through my graduate studies and research. Dr. Donnelly has been supportive of my interest and doings for graduate studies at UVM from the end of my bachelor's degree to the end of my master's degree. It has been a great pleasure to work with a well-experienced and utmost attentive advisor. I would also like to thank Dr. Marie Limoges for being a competent and collaborative partner throughout our research. The department of Nutrition and Food Science was also a very supportive group of professors and faculty members, thank you to all whom helped me grow for the past 6 years through my undergraduate and graduate studies. Lastly, a sincerest thank you to my family and friends for their patience and endless support while completing my graduate degree.

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Traditional use of Wooden Tools for Cheese Aging

For centuries, wooden tools have been used in traditional cheese making for collecting and transforming milk into artisan cheese. Wooden tools include spoons, vats, molds, cream separators, packaging and most importantly shelves for ripening. Wooden shelves have been one of the most questioned wooden tools used for the production of artisan cheese due to the porosity of these materials and the amount of time cheese interacts with the wooden surface. Approximately 500,000 tons of cheese are aged on wooden shelves annually, 350,000 tons of those cheeses are produced in France under an Appellation d'Origin Côntrolée (AOC) regulation, making the tradition a very popular and significant part to the artisan cheese community within the United States and the European Union (Licitra et al., 2014).

When labeling certain traditional cheeses, they are identified under a geographical indication (GI), the two used are Protected Designation of Origin (PDO) and AOC. These labels recognize the product as being unique because of where it is being produced, which includes the region, country, and specific location (Barham, 2003). PDO is the GI of Italy while AOC is the GI of France. The AOC system is the most stringent but oldest labeling of GI, and is controlled by the state to assure the product was recognized for its territorial and precise rules of production under the GI standards it was made (Barham, 2003). These European cheeses are made under the specifications to keep their originality and tradition of flavor, texture, and structure. PDO/AOC cheeses include Comté, Reblochon, Beaufort, Munster, Cantal, and

Roquefort. The European Union (EU) declares in the PDO that traditional cheeses include spontaneous microflora, avoid the use of commercial starter cultures, and use wooden tools including shelves and vats as a means of preserving the tradition of natural processing (Aviat et al., 2016).

Wooden shelves are significant to traditional artisan cheese making because the material is known to improve the development of natural cheese rinds and organoleptic properties (Coude & Wendorff, 2013). These characteristics are only able to properly develop over that maturation process of cheese. The quality of the milk, starter culture, use of pasteurization or not, and environmental conditions of cheese aging will determine the outcome of the cheese product (Weimer, 2007). The rough and porous properties of wood is actually an advantage to cheese makers when aging cheese on the surface, because wood will absorb and release moisture when necessary, creating a consistent environment for the cheese when aging over a long period of time (Percival, 2014). The wooden surface also allows for natural biofilm formation of cultures within the cheese, the biofilm is made of lactic acid bacteria (LAB), which are known to have anti-*Listeria* properties and allow for bactericidal actions on *Listeria monocytogenes* (Mariani et al., 2011; Aviat et al., 2016).

Like the United States, Europe also has regulations governing materials used for food contact surfaces, Aviat et al., (2016) states that wood as a food contact surface is regulated under Regulation (EC) No 1935/2004 of the European Parliament and the Council of 27 October 2004 on materials and articles intended to come into contact with food, a regulation that repeals Directives 80/590/ECC and 89/109/ECC. This text holds general guidelines for materials and articles intended for food contact surfaces,

specifically Article 3 of this document under the heading materials and articles, suggests what materials are allowed when followed under the company's Good Manufacturing Practices (GMPs). The food contact surface should identify as a "material that does not endanger human health or bring about an unacceptable change in the composition of the food, or bring about a deterioration in the organoleptic characteristics, under normal and unforeseeable conditions of use" (Aviat et al., 2016). There are currently no documented guidelines for specifications on wooden tools and equipment for cheese making in the European Union (Licitra et al., 2014). The only expressed concern in regulations is the efficacy of their cleaning and sanitation procedures after each batch of cheese has been ripened on the wooden shelves.

There are guidelines documented for all international trade under the *Codex Alimentarius* (*Codex*). *Codex* is used to protect consumer health when products from all over the world are sent into commerce as imported goods, with the intended purpose of creating a global reference of food standards and codes of good practice for hygienic manufacture of foods (D'Amico, 2014). Annex I of the *Codex* is related to Article 3 of the European parliament guidelines for materials and articles ((EC) No 1935/2004). This section specifies a list of 17 materials and articles, which include wood, to be subjected to specific measures, but to date specific measures harmonized by all European Union countries have only been adopted for plastics, ceramics, and intelligent materials (Aviat et al., 2016). Wood has only been standardized using scientific and technical support by three countries who use the most wooden materials for packaging, fermentation and cheese aging: France, Germany, and Spain (Aviat et al., 2016). Ripening shelves are not only the significant wooden food contact surface being used in

traditional cheese making. Wooden tools have been used for centuries in traditional cheese making for collecting and transforming milk to artisan cheese. In France specific wooden tools must be used to produce AOC cheeses.

Wooden vats (gerles and tinas) are used in cheese making to inoculate the raw milk with bacterial and yeast/mold cultures, as well as to coagulate the milk to form cheese curds (Scatassa et al., 2015). Traditionally, these vats retain and provide a natural biofilm as starter cultures, and the reaction between the milk's microflora and the vat's biofilm create an efficient "starter factory" (Didienne et al., 2012). These wooden vats are made out of either Douglas fir wood or chestnut wood, depending on the country of origin and region of where the cheese is being produced. Also, the cleaning and maintenance of wooden vats depends on the individual cheese company and their handed down traditional practices.

Italian PDO cheeses require the tina to be made within the Sicilian region out of Douglas fir wood for production of cheeses such as Ragusano, Caciocavello Palermitano, and Vastedda della valle del Belice (Lortal et al., 2009; Scatassa et al., 2015). Certain AOC French cheeses such as Salers require use of a wooden vat known as a gerle (Didienne et al., 2012). The traditional practice of using a wooden vat and keeping the natural biofilm to use as a starter culture is protected by the Commission Regulation No 2074/2005, which allows exemption from the Regulation (EC) No 852/2004 "for foods with traditional characteristics as regard to type of materials of which the instruments and the equipment used specifically for the preparation, packaging, and wrapping of these products are made" (Cruciata et al., 2018).

These two cheeses have different associated risks during manufacture, as both

Ragusano and Salers are raw milk cheeses. The critical control points identified during production are important to highlight, because the characteristics of each cheese present different risks during production, which is particularly important to control. Because they are made in and aged on porous materials such as wooden vats and wooden shelves, the microbiological safety of these cheeses must be monitored (Licitra et al., 1998).

A flowchart of how Ragusano cheese is commonly produced was reported by Licitra et al., (1998). Ragusano cheese begins from raw whole milk pumped into a wooden vat (tina), with no added starter culture, which results in a low acidification rate. The initial temperature of milk in the vat is 34.6°C, and this is increased to 40°C after the addition of rennet to start coagulation. In the first cooking step, the cheese curd is held for 12 minutes at 40°C, and the formation of the cheese is similar to ricotta, where the whey is pressed out, and added back into the vat for a second cooking with a temperature up to 84.4°C, which is higher than standard vat pasteurization temperatures (63°C) (Licitra et al., 1998, IDFA, n.d.). Lastly, the curd is ripened for 24 hr, milled and stretched in hot water (78.9°C) to be formed into rectangular blocks, followed by a final step of salting and brining. The curd is typically brined for 24 hr, although some cheesemakers will leave the cheese in the brine a bit longer to allow for further fermentation to reach a desired pH (Licitra et al., 1998).

The production of Salers cheese follows a process similar to Ragusano, in that it is also made from raw unheated milk that is directly pumped into a vat (gerle), and rennet is added. Once the milk has coagulated and a curd has been formed, the newly made cheese is cut and pressed into blocks for a half hour. Lastly the cheese is left to

allow for maturation and acidification of the milk by lactic acid bacteria. Salers cheese is aged for anywhere from 3-45 months (Donnelly, 2016). There are a few differences in how these cheeses are made, but what they have in common when conducting a hazard analysis is that both cheeses are made using raw milk and are produced in wooden vats. The differences in production of these cheeses are how the curds are formed, the Ragusano cheese is made by heating the curd and stretching it out to create blocks (Donnelly, 2016), whereas the Salers cheese does not have a curd heating step and is cut into blocks before being prepared for the aging cycle. In assessing possible risks associated with production of either cheese, for Ragusano, reusing the whey could become a source of contamination, especially if it is not properly handled when added back to the cheese curd from the vat. In addition, stretching the cheese introduces another handling step, and could also pose a risk for contamination from cheesemakers, however, the use of hot water for stretching likely controls any introduction of pathogens. For example, a study done by Junghee et al., 1998 tested the survival of *L. monocytogenes* during mozzarella stretching using three water temperatures (55 °C, 66°C, 77°C) at 1, 3, and 5 minutes. Mozzarella cheese was inoculated with *L. monocytogenes* inoculum of 7 and 3Log CFU/g. Results showed the 66°C tempered water for 3 minutes delivered a 5 Log CFU/g reduction, and 77°C water temperature for 1 minute resulted in complete elimination of *L. monocytogenes* (7 Log CFU/g to reduction of <1 Log CFU/g) (Junghee et al., 1998). Overall, this research concluded that subjecting curd to either 66°C for 3 minutes or 77°C for 1 minute during curd stretching is effective in eliminating *L. monocytogenes* (Junghee et al., 1998).

There have obviously been questions regarding the safety of wooden vats used

for cheese manufacture and if there is control of the natural biofilm comprised with LAB, while it is being preserved for subsequent batches of cheese. Studies have shown that for Salers cheese, the curd cooking temperature of 40°C, acidic environment (pH below 5), and the competition for nutrients inhibit or prevent the growth of pathogen of concern in dairy and cheese manufacturing (Lortal et al., 2009). Previous studies have identified the composition of the bacterial community associated with these biofilms, as well as documented the prevention of contamination by *L. monocytogenes, Salmonella* and *Staphylococcus* species (Lortal et al., 2009; Didienne et al., 2012; Scatassa et al., 2015; Cruciata et al., 2018). Didienne et al., (2012) used milk inoculated with *L. monocytogenes, Salmonella* spp, and *Staphylococcus aureus* to make experimental Salers cheese in wooden gerles. When the gerles and cheese were sampled, *Salmonella* and *L. monocytogenes* were not detected, either on the gerle surface or within the cheese even after enrichment. For *S. aureus,* a 5 log reduction (from 500CFU/ml) was achieved during cheesemaking (Didienne et al., 2012), and these results were consistent with a previous study by Lortal et al., (2009), where only a few of the tinas used for the study showed detection of low levels of *S. aureus*. These results show that properties of the natural LAB biofilm allow for the pH to drop quickly, which creates an unfavorable growth environment for unwanted pathogens. The diversity and strength of biofilms within a tina or a gerle is promoted by *Enterococcus, Lactobacillus, Lactococcus, and Streptococcus* species (Cruciata et al., 2018).

Even though the biofilms are preserved during cheesemaking, there are further steps cheesemakers take to clean and maintain their wooden vats to replenish the biofilm, which keeps the competition strong. There are different methods used by

French versus Italian cheesemakers for cleaning the tinas and gerles. The French will either wash their gerles with water or whey from the same manufacturing day. Whey is drawn from the bottom of the gerle and used to rinse while scrubbing the gerle. When using water to wash to gerle, 40°C tempered water is used while scrubbing the gerle (Didienne et al., 2012). The Sicilians traditionally manage the cleanliness and control of their tinas by washing the vat with hot deprotenized whey made from ricotta cheese production, or with water. Sometimes they will carefully brush the sides of the vat and leave the tina full with whey for about 12 hours (Scatassa et al., 2015).

Although these are all common and traditional ways the wooden vats are cleaned, there has not been an established standard or Sanitation Standard Operating Procedure (SSOP) to follow, and it cannot be known with certainty that these vats are being adequately cleaned. After SSOPs and verification of sanitation methods, trust in traditional practice and absence of foodborne disease outbreaks are ways to determine if wooden vats are being treated in a safe and assuring manner. There are currently no documented guidelines for specifications of cleaning wooden tools and equipment for cheese making in the European Union (Licitra et al., 2014). The French have a few regulations for “any food” versus “solid food” when it comes to the type of wood being used. Aviat et al., (2016) stated that the French Arrêté of 15 November permits wooden food contact surface materials specifically made from oak, chestnut, ash, hornbeam, and acacia for any food production, and walnut, elm, and poplar for contact with only solid food production. It has been previously mentioned that gerles are most commonly made out of chestnut, and most likely for the reason that milk (being classified as “any food”) is an allowable product to be in contact with this type of wood.

There are guidelines documented by the *Codex* that identify how to maintain a clean and safe production process while not outlining specific standards, but overall GMPs. The *Codex Alimentarius* Commission implemented "*Guidelines on Application of General Principles of Food Hygiene to the Control of Listeria monocytogenes in Ready-to-Eat-Foods*" (D'Amico, 2014). These guidelines can be used for imported products with suggestions for producing foods in a manner where *L. monocytogenes* would not survive or spread throughout the facility during processing. This is only one example of the many issued guidelines given by reputable agencies to provide suggestions and methods producers can use when manufacturing traditional cheese products that do not have specific protocols.

Other suggested guidelines have also been reported by the Swiss Confederation and through their Agroscope Institute for Food Sciences (IFS) in 2014, they stated their opinion of how wooden boards should be used for cheese ripening (Imhof, 2014). These guidelines and their reasoning will be further discussed below.

Licitra et al., (2014) elucidated the beginning of specifications for food contact surfaces by the European Union as early as November of 1976, but wood was not specifically stated until July 1996 where milk-based products were specified by their traditional characteristics and instruments used during production. The legislation stated that regardless of the nature of the material used for food production, it must be constantly maintained in a satisfactory state of cleanliness and regularly cleaned and disinfected (Licitra et al., 2014).

Moving forward to a more permanent rule and specification of food contact surfaces, European regulations finalized a Hygienic Package document; guidelines can

be found in Regulation of (EC) No 853/2004 of the European Parliament of the Council of 29 April 2004 on the Hygiene of foodstuffs (EC, 2004). Specific hygienic rules are defined when producing animal and human food with a designated “raw milk and dairy products” section that states that all tools should be well maintained and easy to clean (Licitra et al., 2014). This still does not specify the use of wood directly but does somewhat imply wood because of its porosity make-up and difficulty of cleaning. France still allows and highly prefers the use of wooden material for their cheese making equipment and utensils. There practices are permitted by the French agency for Food, Environmental Occupational Health and Safety, an agency created in July 2010 as a “public administrative institution reporting to the ministries for health, agriculture, environment, labor, and consumer affairs” (Galic & Forbes, 2014). The mission of this agency is to implement multidisciplinary expertise by scientific research that will protect the public health and environment of food and the workplace (Galic & Forbes, 2014). In the United States (US), regulations specify under Code of Federal Regulations (CFR) 21 CFR 117 Subpart B of the current good manufacturing practices for Equipment and utensils (117.40), food contact surfaces must be corrosive-resistant, made of nontoxic materials and able to withstand the processing environment of production, cleaning and sanitizing procedures (FDA CFR, 2018b).

United States Approach to Regulation of Wooden Shelves

Wood has been supported as a surface that is cleanable and safe when properly sanitized, but when cheese manufacturers are using these wooden shelves in rotation for cheese batches there must be a verification step to measure the adequacy of their cleaning and sanitation protocols. The FDA defines adequate as a "means that which is needed to accomplish the intended purpose in keeping with good public health practice" (FDA CFR, 2018b). The artisan cheese industry follows guidelines of FDA's CFR to achieve an acceptable standard of cleaned food contact surfaces which can be found in the 21 CFR 117 Subpart B under "Current good manufacturing practices in manufacturing, packing, and holding human foods" (FDA CFR, 2018b). These guidelines define when food contact surfaces should be cleaned and sanitized and to what standard. Under Part D, "Sanitation of food-contact surfaces" the section states that all food-contact surfaces, including utensils and food-contact surfaces of equipment, shall be cleaned as frequently as necessary to protect against contamination of food. The regulation also states that all food contact surfaces that are wet-cleaned and sanitized must be completely dry in time for production of low moisture foods (FDA CFR, 2018b).

When wooden boards are being cleaned and sanitized for the next round of cheese ripening, verification steps should be taken place to insure they are adequately cleaned to produce safe and quality cheese throughout its aging process (Licitra et al., 2014). Wooden boards are not only used to hold cheese during a set period of time, they are also used to ensure maturity, texture, flavor, and traditional character to the

cheese (Aviat et al., 2016). Any undesired bacteria, yeast, and/or molds left over on these shelves could cause a change and failure of the expected cheese being ripened (Guillier, Stahl, Hezard, Notz, & Briandet, 2008).

FDA expects artisan cheesemakers to implement specific Standard Sanitation Operating Procedures (SSOPs) of their wooden shelves, in addition to monitoring SSOPs to ensure that the process of cleaning and sanitation is adequately done. Sanitation is defined by the FDA as adequately treating cleaned surfaces by a process that is effective in destroying vegetative cells of pathogens, and in achieving substantial reduction of numbers of other undesirable microorganisms without adversely affecting the food product quality or its microbiological safety for consumers (FDA CFR, 2018 b). During SSOPs, cleaning of equipment comes before the sanitation process. Cleaning and sanitation have their own meaning and different terms are associated when forming procedures for both operations. Cleaning is the removal of visible food soil in the action of mechanical cleaning, clean-in-place, and manual cleaning procedures (Schmidt, 1997). Sanitation is known by three terms: sanitize, disinfect, and sterilize (Schmidt, 1997). Schmidt, (1997) defines all three terms: “Sanitizing is done to reduce microorganisms to a level considered safe for consumption, sterilization is a statistical destruction of all living organisms, and disinfection removes all vegetative cells (not spores)” (Schmidt, 1997). Out of these three terms of sanitation, sanitize is primarily used in the food industry. Sanitation and disinfectant practices where first regulated by the Environmental Protection Agency, which defines sanitation by reduction of bacterial numbers to a level considered safe (99.999%) and disinfectants, which removes bacteria on only non-porous surfaces by 99.999% (Leonard et al., 2013). The

difference between the two is the effectiveness on certain types of surfaces, disinfectants will not remove bacteria on porous surfaces (Leonard et al., 2013).

In March of 2014 the FDA announced new guidance for food contact surfaces, stating that wooden shelves, and other "rough" cheese ripening surfaces do not meet the cGMP requirements stated in (21CFR110.40(a)) *"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"*. The American Cheese Society spoke on behalf of the U.S and EU artisan cheese communities in a rebuttal to the FDA's statement, with the correspondence encouraging the FDA to revise the new implemented guidance (21 CFR 110.40(a)) and permit properly maintained, cleaned, and sanitized plant equipment, which includes wood (as an acceptable food contact surface in cheesemaking) as has been enforced by state and federal regulators and inspectors (ACS, 2014). The FDA finally responded within three months (June 2014) and retracted their statement about the wooden boards being "banned" for use in cheesemaking. (USFDA/CFSAN, 2014). The FDA clarified its position on the use of wooden boards in cheese aging, writing that the regulatory agency will act on engaging more with the artisan cheese community, to further understand their traditional practices for future improvements of safety guidelines, which will meet all cheesemaking needs of production (USFDA/CFSAN, 2014).

Wooden shelves have a role in cheese making because they add to the character of the cheese, facilitating natural rind development, and they can tolerate the cheese cave conditions. There is concern that any bacteria including pathogenic bacteria introduced to the food contact surface during production is capable of penetrating 1-2 cm depth into the porous material of the wooden shelves (Mariani et al., 2007).

Sanitation protocols should be designed to ensure complete destruction of any bacterium within the wooden board, not just the topical surface. In addition, cheesemakers must conduct routine environmental surveillance to insure the microbiological safety of wooden boards used for cheese aging.

Significance of Food Safety Plans

Significance of Environmental Monitoring Programs

The success of an environmental sampling program depends on the effectiveness of the detection method used (D' Amico & Donnelly, 2009). *L. monocytogenes* is found regularly in dairy processing facilities, which specifically for this study will include artisan cheese manufacturing facilities. It is important for artisan cheese makers to have stringent environmental monitoring programs (EMPs), in order to control the cleanliness and safety of their facility and finished products. Surveys have documented the presence of *L. monocytogenes* in 33.3% of tested environmental sites in dairy processing facilities (Kathariou, 2002). About 32 strains have been isolated and analyzed from food processing environments of milk and vegetables, and many strains of the 32 were identified as having the ability to form biofilms on stainless steel and glass surfaces depending on their environmental conditions (temperature, surface material, etc.) (Buchanan et al., 2016). *L. monocytogenes* is the main pathogen of public health concern in cheese production, and the most common source of this pathogen is the food processing environment (Buchanan et al., 2016).

EMPs must be designed to effectively monitor, detect, and control the presence of any unwanted microorganism in a food processing environment, and cheesemakers especially should have an aggressive *L. monocytogenes* control program within their EMP, which includes extensive testing to recover *L. monocytogenes* contamination around their facility (Tompkin, 2002; Channaiah, 2013; Beno et al., 2016; Malley et al., 2015). The Food Drug Administration (FDA) created rules for implementation of the

Food Safety Modernization Act (FSMA) (U.S FDA, 2018a), which was signed into law in 2011 by President Obama. This act required all FDA-regulated facilities that produced ready-to-eat (RTE) food products to have a food safety plan (Beno et al., 2016).

FSMA has multiple rules used by the industry for suggested guidelines to follow when creating food safety plans. One in particular, that is relevant to this thesis research is the Preventive Controls for Human Food (U.S FDA, 2018a). Processing facilities that are mandated under this rule must have a hazard analysis of their production, preventive controls for those hazards, monitoring programs, corrective actions, and verification methods to check efficacy of those corrective actions (U.S FDA, 2018a). , All facilities that produce ready-to eat-food (RTE) under the regulations of FSMA must follow various subsections in 21 CFR 117, depending on the risk of the product being made (U.S FDA, 2018a). Both domestic and foreign, to register and comply to all requirements of a risk-based preventive controls of FSMA and formulate current GMPs under section 415 of the Food, Drug, and Cosmetic Act (U.S FDA, 2018a). Facilities that do not produce RTE food products typically do not need to create the common environmental monitoring programs, which are used to assess how adequately a facility and its employees are following their GMPs, monitoring pathogenic contamination and the efficiency of their sanitation standard operating procedures, the program is ultimately used to monitor effectiveness of the preventive controls (Channaiah, 2013). The EMP is created based on the type of raw materials coming into the facility and how the product(s) is produced, not all EMPs are created equal and are customized to fit the facilities production. The cheese industry's raw materials primarily include pasteurized or raw milk, which are main concerns for pathogens contamination, especially raw milk products. Raw milk carries a

high microbial load of bacteria, which may include pathogenic bacteria such as, positive *Staphylococcus, Corynebacterium diphtheriae, Streptococcus pyrogenes, B. cerus, and Campylobacter jejuni*, and lastly but most well-known is *L. monocytogenes,* which can lead to persistent contamination (Ibba et al., 2013). The quality of milk is where the safety of cheese starts (D'Amico, 2014).

Depending on the product and raw materials used to make the product, specific pathogen monitoring and identification will be chosen accordingly. The most significant aspect of the EMP includes designating the zones of a processing facility to be monitored and sampled, along with the frequency of sampling (Channaiah, 2013). The zones are identified numerically from one to four, where zone one sites are surfaces and tools that come into direct contact with in-process and finished product. Zone 2 sites are surfaces and equipment physically close to exposed product, zone 3 sites are surfaces and equipment within general vicinity of product (drains, walls, floors), and zone 4 sites are outside of exposed product areas (Beno et al., 2016; Channaiah, 2013). This thesis research study was mainly concerned with environmental sampling of zone one sites (food contact surfaces) within an artisan cheese production facility, including wooden shelves for cheese aging.

An environmental monitoring program is unique based on the facility and the product being manufactured. Malley et al., (2015) suggest using three fundamental steps to seek and destroy bacterial contamination when sampling environmental surfaces and zones- (i) verification, (ii) process control, and (iii) investigate. When starting with verification, the sampler will seek out zone 1 (food contact surfaces) as well as zones 2 and 3 for pathway verification. This sampling should be done while production is in

process, to increase detection of pathogens while they can be mobile and move through all steps of production. Process control is used to locate indicator sites using zones 3 and 4 because they can be considered transfer pathways. This process should be done post rinse and during the foaming process of cleaning production areas. Lastly, investigative actions can take place when a positive pathogen result is found, and controls in the process can be implemented. The best way to verify process control is to sample post sanitation to ensure the facilities sanitation standard operating procedures are effective.

Carpentier and Cerf, (2011) indicate how harborage of bacteria can travel through the mechanical actions of a production line. Liquids or aerosols are most commonly known for initiating spread of contamination from one zone to another. These harborage sites are known as niches, which are defined as a sheltered site within the equipment of the production line that allows for bacteria such as *L. monocytogenes* to survive and establish even after cleaning and sanitation (Tompkin, 2002; Carpentier & Cerf, 2011). The seek and destroy method described by Malley et al., (2015) is used to find the niches within the production line that could potentially hold harborage of bacterial contamination, or more importantly persisting strains of pathogens such as *L. monocytogenes*. Artisan cheesemakers must identify the areas in their facility that have potential for persistence or sporadic growth of *L. monocytogenes*

There are various methods that can be used when testing environmental samples for *L. monocytogenes*. Each method has the same end result, but different steps are taken to find the result. The FDA's Bacterial Analytical Manual (BAM) (U.S FDA, 2017a) outlines primary enrichment as a three-step, three-day process to recover *L. monocytogenes* from environmental sample swabs. Third party labs or manufacturing

facilities (with the ability to run their own tests) will use a Real Time PCR (polymerase chain reaction) to locate results of environmental samples within 24-48 hours. Examples of PCR assays include the Dupont Bax, which was used in this research and has been used by others (D'Amico & Donnelly, 2008; Lee et al., 2018; Pan, Breidt, & Kathariou, 2006; Scatassa et al., 2015; Zunabovic, Domig, & Kneifel, 2011).

Tools used for Environmental monitoring: Sampling Procedures

An environmental swab is commonly used to test food-processing environments because they are made to pick up, hold, and preserve any viable microorganism on surfaces (Ward, 2013). Swabs can either come in a dry or pre-moistened form. These swabs are typically pre-moistened in a neutralizing broth, used to neutralize any sanitizers left behind on a sampled surface , which may interfere with enrichment and outgrowth of the target bacterium. The type of neutralizing broth is significant because it must neutralize sanitizers from the sampled surface and maintain viable organisms until they are further processed (Ward, 2013). There are various types of neutralizing broth including Dey-Engley (D/E), High Capacity (Highcap), Letheen, and Neutralizing buffer broths and are all commonly used by many food manufacturing facilities (Ward, 2013; Fort, 2011). The FDA BAM (U.S FDA, 2017a) does not support any one commercial company that produces sponge samplers for environmental sampling, but instead list examples of formats that could be used. The FDA BAM recommends use of the 3M™ sponge-sticks soaked in 10ml of D/E broth, as well as the World Bioproducts EZ Reach Sponge sampler also soaked in 10ml of D/E broth (U.S FDA, 2017a). The FDA only suggested one of the four broths (Dey Engley; D/E) made for environmental sampling not

in Highcap, Letheen, or even the standard neutralizing broth (U.S FDA, 2017a). The Food Safety Inspection Services (FSIS) of the United States Department of Agriculture (USDA), does not state any commercial brand of swabs in their guidelines for *L. monocytogenes* environmental sampling detection; they only recommend use of a sterile cotton swabbed tip (USDA/FSIS, 2012).

Two broths used for this study were Dey-Engley and High Capacity. Dey-Engley broth is comprised of Tryptone, Yeast extract, Glucose, Sodium, thioglycolate, Sodium thiosulfate, Sodium biosulfate, Polysorbate 80, Lecithin (soy bean), Brom cresol purple, and Distilled water (U.S FDA, 2005), versus High Capacity is a newly developed broth and only available by commercial prepared tubes and pre-moistened sponges (Ward, 2013). Dey-Engley and High Capacity broths are known to perform well with all sanitizers used in the industry, Letheen and neutralizing buffer do not work with high acid and quaternary ammonium compound sanitizers (Ward, 2013; Zhu et al., 2012).

Commercial swab and sponge formats are available from World Bioproducts and 3M™. Both companies use the same type of swabbing tool and size of sponge, but each sponge is made from different materials. 3M™ uses a cellulose sponge and World Bioproducts use a polyurethane material. The polyurethane material is known to be stronger and more resistant to tearing, flaking and fraying (Fort, 2011; World Bioproducts, n.d.). Polyurethane is manufactured without toxins such as quaternary ammonium compounds which could cause inhibition of growth when microorganisms are picked up by the sponge swab (Fort, 2011; World Bioproducts, n.d.). The 3M™ sponge made with cellulose, the most common material used for environmental sponges. Cellulose is known to be manufactured with toxic materials such as quaternary

ammonium compounds, which could leave chemical residues within the sponge leading to growth inhibition of isolated microorganism during environmental sampling (Daley, Pagotto, and Farber, 1995; Fort, 2011). Fort, (2011), reported that cellulose sampling sponges can break apart, leaving small pieces onto swabbed rough and sharp surfaces.

The Challenges with Sampling Wooden Shelves

Wood can be a challenging food contact surface during environmental monitoring, because of the porosity, roughness, dryness, and irregular state of the board (Ismail et al., 2017). Monitoring wooden boards after sanitation is vital to verify if cleaning and sanitation protocols were successful. A study conducted by Zangerl et al., (2010) documented cleaning and sanitation protocols for use on wooden shelves after each ripening cycle to destroy *L. monocytogenes.* These authors found that wooden shelves soaked in a hot alkaline solution (50°C) for 15 minutes, brushed for 30s, rinsed with hot water (50°C), and either be heated for 5 minutes under 80°C or 15 minutes under 65°C effectively destroyed *L. monocytogenes* (Zangerl at al., 2010). This study used an initial inoculum of $5.5x10^7$ CFU *L. monocytogenes* on wooden boards treated only with soaking and brushing the boards; versus soaking, brushing and employing a heat treatment. They found that *L. monocytogenes* was only effectively destroyed by heat treatment. Zangerl et al., (2010), also mentioned that cheesemakers in many other countries use this method of a heat treatment or desiccation process to destroy *L. monocytogenes* on their wooden shelves post ripening. Other studies have reported similar results (Imhof et al., 2017; Mariani et al., 2011).

Mariani et al., (2011) compared the survival of *L. monocytogenes* on wooden

boards with a naturally preserved biofilm versus heat treated wooden boards. Initial populations of *L. monocytogenes* applied to boards remained stable or decreased by 2 $\log_{10}$(CFU/cm^2) following 12 days of incubation at 15 °C in the presence of biofilms. In contrast, when the resident biofilm was inactivated by heat treatment, *L. monocytogenes* populations increased up to 4 $\log_{10}$(CFU/cm^2) when the resident biofilm was thermally inactivated. This research suggests that control of pathogens such as *Listeria* on wooden shelves by resident biofilms could enhance the microbiological safety of traditional ripened cheeses.

Imhof et al., (2017) reported the effects of steam treatment of wooden surfaces for *Listeria* spp. decontamination. Initial populations of *L. monocytogenes* and *L. innocua* (2.6Log CFU/cm^2 , 4.6Log CFU/cm^{2,} respectively,) were fully decontaminated on wooden surfaces when held at 70^{o}C 20 minutes (Imhof et al., 2017).

French versus Swiss approach to Clean/Sanitize Wooden Shelves Post Aging Cycle

French and Swiss cheese makers utilize different protocols for cleaning their wooden shelves post ripening as a result of their traditional cheese making practices. The French approach involves maintaining a healthy biofilm on wooden shelves, to not only improve the flavor and characteristics of the cheese rind, but to outcompete *L. monocytogenes* on the boards (Oulahal et al., 2009). The French cleaning protocol includes brushing the boards for three seconds, three consecutive times with cold water, and placing cleaned boards in an air drying system (Oulahal et al., 2009). However, if the boards experienced overgrowth with pathogenic bacteria, the process of sanitation is not enough to completely destroy the pathogens throughout the entire board, causing a

facility to discard contaminated boards and start with new ones (Coude & Wendorff, 2013).

The Swiss approach involves a more sterile approach when cleaning and sanitizing wooden shelves post ripening. During 1983, a large listeriosis outbreak occurred in Switzerland with 31 fatalities, this caused the Swiss cheese makers to practice more sterile cleaning procedures for their wooden boards (Ferreira, Wiedmann, Teixeira, & Stasiewicz, 2014). Agroscopes Institute for Food Sciences (IFS) was given the responsibility to create a process to monitor and control *L. monocytogenes* during cheese manufacturing. IFS was successful in developing a system that Switzerland uses today, which includes mechanical cleaning with alkaline detergent at 60°C and rinsing with water, or spraying the boards with stream with normal pressure, to achieve an internal temperature of >70°C for 30 minutes (Imhof et al., 2017; Imhof, 2014) Like the French, they also use a closed drying method to ensure complete destruction of surviving microorganisms.

Pros of the Property of Wooden Shelves

There are a few properties of wood that separates itself from other food contact surfaces used in the food industry. The porosity and hygroscopicity of wooden shelves makes the material one of the best for aging cheese. During cheesemaking there is a high degree of moisture within the environment of the aging caves (98% RH), and wooden boards are able to hold and release a considerable amount of water based on the environment relative humidity (RH). For example at 80% RH, wood is able to uptake

14% of that moisture (Aviat et al., 2016). This is significant when aging cheese because these wooden shelves are able to adapt to the environmental conditions and create a perfect microclimate for the cheese. When using plastic or stainless-steel shelving, the rind of the cheese can become too moist due to condensation left over from the cave's moist environment, creating an overly-soft cheese, which becomes more susceptible to spoilage microbes and decreased shelf life (Aviat et al., 2016). Wood is also known to have natural antimicrobial properties. According to (Zangerl et al., 2010), microorganisms were found within the inner parts of the wood and on the outside of some cheese rinds, including salt tolerant micrococci, corynebacterial, yeasts, and molds as dominant microflora.

A study conducted by Ismail et al., (2017) showed transfer rates of *L. monocytogenes* from wood and plastic surfaces to young cheese during aging conditions. Results showed that when inoculating wood and plastic with high concentrations of *L. monocytogenes,* the transfer rate from the inoculated surface to cheese was 1.09% for plastic versus 0.55% for wood (Ismail et al., 2017). This study was able to prove that wood may be a porous and hard to clean contact surface, but when contaminated with *L. monocytogenes*, the transfer rate is significantly slower than the transfer rate of plastic surfaces.

There are many types of wood used not only for cheese making, but throughout the food industry, and the survival of bacteria may depend on the species of wood being used (Schönwälder, Kehr, Wulf, & Smalla, 2002). Schönwälder et al., 2002 assessed the antibacterial properties and survival of bacteria on pine, spruce, beech, poplar, and larch boards, using *E. coli* species as the pathogen of concern. Even though this study did not

assess survival and sanitation of each species of wood, Schönwälder et al., (2002) stated that some wood species may have antibacterial properties and could out compete unwanted bacterial pathogens during cross-contamination. Because of the porous and hydroscopic characteristics, wood is also able to remove water the pathogen needs to survive, which would cause a reduction of bacteria. Pine, oak, and larch are wood species known to have better hygienic characteristics than other species of wood, and this study was done to investigate that hypothesis (Schönwälder et al., 2002). There were two methods of inoculation used, topical surface inoculation and 15 minutes of soaking the wooden surfaces in an inoculum, to allow absorption into the deeper layers of the board (Schönwälder et al., 2002).. This was done to compare the recovery not just on top, but to see if the inner chemical properties of the wooden board have antibacterial properties or not.

The overall results showed high recovery rates of bacterial colony forming units (CFU) on the surface and within the inner structure from beech and poplar (Schönwälder et al., 2002). Spruce results showed high recovery of CFUs, meaning limited antibacterial properties (Schönwälder et al., 2002). This study was able to support the hypothesis of pine being one of the most antibacterial species of the wood being tested, because the recovery decreased over the sampling time, which means the survival of *E. coli* were also decreasing. In general the absorption and transfer of the bacteria within the layers of a wooden board will determine the survival and translocation of contamination from the surface (Schönwälder et al., 2002).

When harvesting wood used to make shelves for aging cheese, the process takes time and is seasonally dependent (Coude & Wendorff, 2013). Wood is harvested in the

winter months because the sap has moved from the top to the bottom of the tree, and the best part of the tree used for durable, long lasting shelves are boards cut close to the heart of the tree (Coude & Wendorff, 2013). Once the wood has been cut into shelves, boards must be "seasoned" for about 18 months, allowing all bound moisture and yeast or molds to dry out. Extra microorganisms or moisture within the shelves could negatively affect the cheese if boards are not seasoned properly; these boards must be prepared as a clean slate before being used for aging young cheese (Percival, 2014).

Spruce and beech woods are known to be the most common wood species used for cheesemaking, because their properties allow for development and preservation of natural biofilms during the aging period (Aviat et al., 2016). The literature does not specify a single wood species to be used to age cheese. There are different reasons why cheesemakers use different species of wood, those factors including the region they are located in, traditional practices learned from previous cheesemakers they have worked with, and seasons of the region the wood resides in. Spruce was used in this thesis study because it was readily available and most commonly used in scientific studies that also research the safety of wooden shelves for cheese aging.

Pathogens of Concern for this Study

Listeria monocytogenes

L. monocytogenes is a Gram-positive, rod shaped bacillus that is facultatively anaerobic and an intracellular, non-spore forming pathogen (Thakur, Asrani, & Patial, 2018). *L. monocytogenes* is highly prevalent in agricultural environments that include soils closer to water, cultivated soils, pastures, and land that is irrigated or consistently rained upon (Buchanan et al., 2016). Food is a common vehicle for *L. monocytogenes,* which leads to the high rate of listeriosis infections in the U.S (Buchanan et al., 2016). This pathogen is known to be a major foodborne pathogen of concern and has been the cause of many foodborne epidemics associated with various ready-to-eat foods (RTE), especially dairy products. Environmental conditions such as temperature, pH, water activity, and salt concentration can either influence or suppress the growth of *L. monocytogenes* (Dortet, Veiga-Chacon, & Cossart, 2009). The temperature growth range of *L. monocytogenes* is from -1.5-45°C, with an optimal growth temperature of 30-37°C (Dortet et al., 2009). *L. monocytogenes* can also thrive in an acidic environment, surviving within a pH range of 4.0-9.6 (Dortet et al., 2009). Like all bacteria, higher water activity (A_w) can influence the growth of *L. monocytogenes*, for example 0.97 A_w is an optimal A_w level for *L. monocytogenes* growth, however, *L. monocytogenes* also has the ability to survive in conditions with lower water activity such as 0.90 A_w (Dortet et al., 2009). Lastly, *L. monocytogenes* can grow within some food matrices having salt concentrations ranging from 13-14% (Dortet et al., 2009)

L. monocytogenes is able to adapt to its environment and has shown the ability to

develop resistance to quaternary ammonium-based sanitizers. Recent outbreaks have shown a linkage between ST6 strains harboring quaternary ammonium resistance conferred due to uptake of a bacteriophage that also leads to gentamicin resistance (Kremer et al., 2017). These environmental growth conditions previously explained by Dortet et al., (2009) are the reason why *L. monocytogenes* is one of the leading pathogens of concern in the food industry, especially to those facilities producing ready-to-eat foods, and other raw food ingredients such as milk, produce, and meats (Dortet et al., 2009). This pathogen was chosen for this study because of its prevalence within the dairy foods industry, raw milk, raw milk cheeses and even pasteurized dairy products, which are all at high risk for contamination by *L. monocytogenes*.

Three specific strains of *Listeria* species were chosen for this study, two *Listeria monocytogenes* strains and one *L. innocua* strain, all chosen based on their prevalence within the dairy industry. The two *L. monocytogenes* strains include ATCC® 19115™ and DUP-1042B, both 4b serotypes which are known to be the most common cause of human infections (Ooi & Lorber, 2005). The *L. monocytogenes* ATCC® 19115™ strain is a clinical strain isolated from human source and used for research-laboratory purposes (Murry et al., n.d.). *L. monocytogenes* strain DUP-1042B, a lineage I subtype commonly associated with human clinical isolates was used because it is commonly found within dairy farms and shows environmental persistence. This strain was isolated by the Donnelly laboratory at the University of Vermont and identified as (CW 193-10 M5-1).

Listeriosis

L. monocytogenes is a pathogen that is normally transmitted from the environment to the victimized host (Nightingale et al., 2004). Ingestion through a port of entry is the start of infection, which is most likely to be a source of contaminated food or water (Nightingale et al., 2004). *L. monocytogenes* causes listeriosis, an invasive disease in humans and animals with symptoms such as febrile gastroenteritis, perinatal infection, and systemic infections leading to death (Drevets & Bronze, 2008). The U.S. Centers for Disease Control and Prevention (CDC) states that it is common that symptoms from listeriosis may not be present or reported until 1-4 weeks post ingestion (CDC, 2017a). Some cases are reported as late as 70 days post exposure, and others as early as same day exposure (CDC, 2017a). The incubation period and development of listeriosis depends on the dose of cells ingested from the contaminated food or water. Listeriosis is known to affect mostly a susceptible population, more so than a person with a healthy immune system. When immunocompromised individuals are infected with listeriosis, they are commonly diagnosed with sepsis, meningitis, and meningonencaphalitis (CDC, 2017a). Individuals with a healthy and strong immune system do not normally contract an invasive form of listeriosis, but when there is a case of listeriosis within a relatively healthy individual, it involves symptoms of self-limiting acute febrile gastroenteritis, which is mostly caused by a high dose of *L. monocytogenes* (CDC, 2017a).

CDC reports that *L. monocytogenes* causes ~1,600 illnesses and ~260 fatalities each year (CDC, 2017a; Scallan et al., 2011). *L. monocytogenes* is also ranked as the

third or fourth most common cause of bacterial meningitis within North America and Western Europe (Drevets & Bronze, 2008). Pregnant women are most susceptible to a listeriosis infection and account for about 27% of all infections reported (Delgado, 2008). *L. monocytogenes* intracellular transmission mechanisms allow for the bacteria to cross various tissue barriers and one barrier it can cross is the placental barrier (Delgado, 2008). When a pregnant woman becomes infected with listeriosis, the pathogen can infect both mother and fetus.

Outbreaks Associated with *Listeria monocytogenes*

Although most cases are sporadic, *L. monocytogenes* is a foodborne pathogen that has been the cause of many multistate foodborne outbreaks throughout the U.S. A recent outbreak involving Vulto artisan cheese occurred due to *L. monocytogenes* contamination of finished cheese products from a direct food contact surface (zone 1). The outbreak was reported in March 2017, and a New York artisan cheese company, Vulto Creamery, was shut down because of this outbreak, which resulted in 8 cases of listeriosis and 2 deaths in patients from 3 states. Food contact surfaces and finished cheese products were found to be positive, all from poor GMPs and not properly following food safety regulation protocols (CDC, 2017b). A review of the FDA Form 483 issued to Vulto Creamery revealed many sources of contamination around the entire facility, where 54 out of 198 environmental samples taken over a three-year period from 2014-2017 were found to be positive for *Listeria* species (FDA HHS, 2017). The FDA Form 483 also reported 10 of 54 positive *L .monocytogenes* environmental samples were

recovered from food contact surfaces used in the facility, Vulto creamery used wooden shelves to age their cheese and these positive environmental results came from those surfaces (FDA HHS, 2017).

In 2011, a large multistate outbreak involving *L. monocytogenes* contaminated cantaloupes was reported, resulting in 147 cases of listeriosis and 33 deaths in 28 states (McCollum et al., 2013). The outbreak was caused by contaminated conveyer belt causing cross contamination to the cantaloupes (CDC, 2012; McCollum et al., 2013). Another multistate outbreak of listeriosis infected 10 people resulting in 3 deaths was caused by contaminated ice cream produced by Blue Bell creameries. The outbreak lasted from 2010-2015. The Blue Bell ice cream was found to be positive for *L. monocytogenes*, and this ice cream was given to hospitalized cancer patients as milkshakes during their treatment. Temperature abuse and improper cleaning of the equipment that made each milkshake allowed cross contamination of other milkshakes made at the hospital (CDC, 2015; Chen et al.' 2017). The level of *L. monocytogenes* contamination of the ice cream is important because it showed that the contamination was at a low and non-infectious dose for the general public, but when given to a susceptible population it was high enough to cause infection (Pouillot et al., 2016). A study done by Pouillot et al., (2016) estimated a dosage of *L. monocytogenes* in each serving if ice cream eaten by the general public versus hospitalized patients. The dose consumed by the general public was estimated to range from a low dose of $1.5x10^9$ CFU/g to high dose of $1.4x10^{10}$ CFU/g, compared to hospital ice cream servings with a low dose of $7.2x10^6$ CFU/g and high dose of $3.3x10^7$ CFU/g (Pouillot et al., 2016). *L. monocytogenes* is capable of surviving in the cold temperatures of ice cream, but the product does not support growth conditions. It is

possible that the secondary processing of the ice cream into milk shakes for the hospital patients could have allowed for increased growth of *L. monocytogenes,* but because of underlying health conditions even a low cell count of the pathogen can cause a listeriosis infection (Pouillot et al., 2016). These outbreaks show how *L. monocytogenes* can adapt and survive in all different food matrixes, from cantaloupes to ice cream, to cheese.

The ability of *L. monocytogenes* to evolve and adapt to its environment over time has been increasingly recognized as we continue to use advanced investigative tools such as Whole Genome Sequencing (WGS) on the strains recovered from outbreaks. A study by (Chen et al., 2017) identified three multistate outbreaks linked together by the same *L. monocytogenes* clonal group of a singleton sequence type 382 in stone fruit, caramel apples, and leafy green salad., The isolates of the ST832 *L. monocytogenes* were identified as serotype 4b (Chen et al., 2017). Chen at al., (2017) was able to calculate the diverging timeline of the ST832 strains, it was found that all strains from each outbreak were emerging for the last 18.4 years. Lee et al., (2018) in a related study, reported the emergence clonal complexes (CC's) CC1, CC2, CC4, and CC6 and their association with food, human, and environmental prevalence (Lee et al., 2018). These authors found the pathogenicity of the clonal *L. monocytogenes* groups have increased and enhanced their capability of placental and neurovirulence (Lee et al., 2018). This supports the case for *L. monocytogenes* evolution and emergence to as an increasingly dangerous pathogen in our food system.

Listeria innocua

L. innocua is a non-pathogenic bacterium commonly found in the environment of food processing facilities alongside *L. monocytogenes.* As a non-pathogenic strain, *L. innocua* does not cause infectious disease in humans when ingested, due to its lack of Listeriolysin O (LLO) that allows the colonization of *L. monocytogenes* in the liver or spleen, which is necessary for infection and pathogenicity (FAO/WHO, 2004). *L. innocua* is an indicator of the presence of *L. monocytogenes*. A specific *L. innocua* strain was use for this study, *L. innocua* 18 a non-pathogenic strain isolated from a food processing environment (Ma, Zhang, & Doyle, 2011). This strain was chosen because it possesses a gene encoding for green florescent protein, constructed by an academic lab at the Oklahoma State University (Ma, Zhang, & Doyle, 2011). In previous studies, *Salmonella*, *Listeria*, and *E. coli* species expressing a green florescent protein were used to study gene expression and monitor protein localization in live cells (Ma, Zhang, & Doyle, 2011). The GFP strains are able to be seen under long UV wavelengths, and used at any growth phase of the bacterium (Ma, Zhang, & Doyle, 2011). The *L. innocua* expressing GFP was constructed by tagging vector gene *gfp-mut1* to transcribe *L . monocytogenes* promoter gene *Pdlt*, the vector gene (*gfp-mut1*) was tagged with a plasmid pNF8, derived from an *E. coli HB101p* strain (Ma, Zhang, & Doyle, 2011).

The reason behind choosing a GFP-expressing *L. innocua* strain for this thesis research was to observe the strain on environmental surfaces under a fluorescence microscope, and to visualize the difference between the performance of each chosen environmental sponge swab for *Listeria* recovery. Environmental surfaces were

inoculated with the GFP *L. innocua* 18 strain and an image was taken before and after the swab was used. Images were then analyzed based on the movement of the bacteria before and after the swab was used. This strain did not only give us the ability to study the strain through a qualitative visual perspective, but it also gave diversity to the strains chosen for this study because it was a non-pathogenic *Listeria* species, commonly found within food processing and farm environments. This GFP strain has been used by other studies for similar reasonings, to visually see the presence or absence of *Listeria* during real time contamination, for example a study has been done to observe the presence or absence of *Listeria* GFP strains during an aerosolized study on meat. The study consisted of allowing the *Listeria* GFP strains to be aerosolized in a chamber over open petri dishes of various meats, ultimately to see a visual of the contamination results. The idea was to use the florescence in the study, but the GFP strains were not utilized for showing presence of contamination (Zhang, Ma, Oyarzabal, & Doyle, 2007).

***Listeria innocua and Listeria monocytogenes* survival competition**

When monitoring *L. monocytogenes* in food products or processing environments such as dairy processing facilities, *Listeria* species are just as commonly found, especially *L. innocua* (Zitz, Zunabovic, Domig, Wilrich, & Kneifel, 2011). It has been reported that *L. monocytogenes* serotype 4b strains may be sensitive to the presence of *L. innocua* and could be the reason why serotype 4b is not always found in food samples and environments (Zitz et al., 2011). There is concern that the detection of *L. innocua* in an environmental sample can mask the presence of *L. monocytogenes*,

especially when running methodologies that identify *Listeria* species, not specifically *L. monocytogenes* (Tompkin, 2002; Zitz et al., 2011). When working to detect *L. monocytogenes* from an environmental sample, these methods use selective agents in an enrichment method to only allow growth of *L. monocytogenes,* but some selective agents such as acriflavine are known to act negatively towards *L. monocytogenes* (Zitz et al., 2011), inhibiting repair of *L. monocytogenes* 4b serotypes. Acriflavin was not found to influence the growth of *L. innocua* in either a negative or positive way (Zitz et al., 2011).

US and EU Limits of Pathogens of Concern

The pathogens of concern in cheesemaking and aging can arise from contamination by feces and bedding material that get onto the teats of the cows and are released into the raw milk during milk processing (D'Amico, 2014), or through ingestion of contaminated silage (Ho, Ivanek, Gröhn, Nightingale, & Wiedmann, 2007). In addition to *L. monocytogenes*, there are additional pathogens of concern for cheesemakers, which include *S. aureus*, *E. coli* (O157:H7 and other STECs), and *Salmonella* spp. U.S FDA and the European Union (EU) regulations have established microbiological criteria for cheeses and limits for these pathogens. In the U.S., the FDA has established a zero-tolerance policy for *L. monocytogenes* in RTE food products including cheese (Archer, 2018). Zero tolerance is defined as less than 0 cfu/25g of tested RTE product. The EU has established tolerance limits for *L. monocytogenes*, with a food safety objective target of less than 100 cfu/25g at time of consumption (EC, 2005).

Escherichia coli contain pathogenic and non-pathogenic members. Pathogenic

species of concern are known as STEC *(*Shiga toxin-producing *Escherichia coli)* and are able to survive during cheese manufacture (D'Amico, 2014). STEC strains of concern in cheesemaking include O157:H7, O26:H11, O111:H8, and O145:H28. Set by the U.S FDA (2008), a food with detectable levels of *E. coli* O157:H7 is considered adulterated and will not be allowed to enter commerce. Illness from Shiga toxin-producing *E. coli* can result in hemolytic uremic syndrome, non-bloody diarrhea, and hemorrhagic colitis (Ryser, 2001). EU Microbiological criteria for cheese and milk intended for cheesemaking are different depending on whether the cheese has been made from raw or heat-treated milk. For cheese made with heat-treated milk, the EU has established limits for *Staphylococcus aureus* as their food safety criteria, as well as with targets for *S. aureus* and *E. coli* as hygienic criteria (EU, 2005). *E. coli* limits are scientifically meaningful standards in cheese made with heat-treated milk because *E. coli* will not survive heat treatment. The presence of *E. coli* in cheese made from heat-treated milk therefore indicates post-process recontamination. In comparison, for raw milk cheeses, microbiological criteria for coagulase-positive *Staphylococcus* are provided, where n=5, c=2, m=10^4 and M=10^5. Cheese should be tested at the time during cheesemaking when coagulase positive *Staphylococci* are expected to be at the highest levels.

In soft and semi-soft cheeses, the majority of *S. aureus* growth occurs from inoculation to salting, so the curd should be tested (Cretenet et al., 2011). In the case of unsatisfactory results, production hygiene and raw material selection should be improved. If *S. aureus* levels exceed >10^5 cfu/g, the cheese batch must be tested for staphylococcal enterotoxins.(EC, 2005). Coagulase-positive *S. aureus* produces a heat-stable enterotoxin that when ingested by human or animals can cause illness. Most outbreaks that include

Staphylococcus are linked back to either mastitis within the herd of cows or a post pasteurization-post processing contamination from human handling leading to consumer illness (D'Amico, 2014; Cretenet et al., 2011).

Within the *Salmonella* species, *Salmonella enterica* is the most common subspecies that holds the largest number of serotypes and causes the most human infections. *Salmonella* can cause a range of human illnesses from gastroenteritis to typhoid fever, with severity of the infection dependent on the host susceptibility, number of cells, and the type of strain. Common *Salmonella* isolated strains from contaminated foods are *S.* Typhimurium, *S. Newport*, *S. Heidelberg*, and *S. Montevideo*. These strains were reported to cause 89% of reported salmonellosis infections from 2007-2011 (Andino & Hanning, 2015). Although *Salmonella* is known to be the leading cause of foodborne illness, 70% of the outbreaks are associated with consumption of contaminated eggs, turkey, and chicken (Andino & Hanning, 2015). The pathogen can be found in animal's intestine and cause contamination from feces to milk. Most dairy related outbreaks are caused by raw milk and inadequately pasteurized milk (D'Amico, 2014). Regulation EC 2073/2005 requires absence of *Salmonella*/25g cheese at end of production (EOP) and during shelf life (FSAI, 2016). In the US, if *Salmonella* is found in processing facilities, a 5-log reduction of the detected positive area or equipment that was sampled must be shown (U.S FDA, 2011).

Methods for Detection of *Listeria monocytogenes* in Dairy Processing Facilities

Conventional Methods

Methods of Plating

Plating techniques for food samples and bacterial cultures are used for many purposes including colony quantification, assessment of growth characteristics, and presence or absence of growth in an environmental sample. There are various plating media used to detect and select for *L. monocytogenes* when investigating environmental and food samples. These agar media are developed to inhibit the growth of background microorganisms using selective agents such as antibiotics including acriflavine, nalidixic acid, and cycloheximide (Farber & Peterkin, 1991). Differential agars used for *L. monocytogenes* and *Listeria* species have either an esculin base or a chromogenic base. Esculin containing media recommended by the FDA BAM are Oxford agar (OXA), modified Oxford agar (MOX), PALCAM, and LPM (U.S FDA, 2017a). The chromogenic base agar media are used for selecting *Listeria* species and distinguishing between *L. monocytogenes* and *L. ivanovii*. These agars are R&F *Listeria monocytogenes* Chromogenic Plating Medium (R&F LMCPM), RAPID' *L. mono,* Agar *Listeria* according to Ottaviani and Agosti (ALOA) or Oxford *Listeria* agar (OCLA), and lastly CHROMagar *Listeria* (U.S FDA, 2017a). Chromogenic agar can be used as a selective and differential medium, for example this research used the media as a differential medium for *L. monocytogenes.* To select for different pathogens like *L. monocytogenes*, the medium is made with a specific synthetic chromogenic enzyme substrate (5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside, X-glucoside) which targets desired pathogen of

choice (Law, Mutalib, Chan, & Lee, 2015; Perry, 2017). Chromogenic agar is known for the color detection used for identifying pathogens colonies on the medium, the color of the colonies is possible by manipulating the metabolism and hydrolysis of the enzyme substrate designated for the pathogen the chromogenic agar is being produced for (Perry, 2017). In the case of *L*. monocytogenes, the pathogen is identified by a metallic blue colony surrounded by an opaque halo, this is made possible by the media being designed to react to the production of Phosphatidylinositol-specific phospholipase C (PI-PLC) encoded by virulence gene plcA which is specific to *L. monocytogenes* (Law et al., 2015). The specific virulence gene plcA and PI-PLC is hydrolyzed by L-α-phosphatidylinositol which is a significant competent to the media and allows for selective detection of *L. monocytogenes* (Law et al., 2015).

Plating can also be done by dry media culture plates such as Petrifilm™. These plates can be used for non-selective and selective growth of *Listeria* in many settings such as predicting shelf life and monitoring the quality of a wide range of foods. Petrifilm™ plates are developed using layers of plastic film which encase a thick paper material with dehydrated media appropriate to its designated purpose (coliform testing, aerobic bacterial count, etc) (Gracias & McKillip, 2004). A selective dry culture medium used for detecting *L. monocytogenes* is 3M™ Petrifilm™ Environmental *Listeria* plates. These plates are used as an inexpensive and rapid testing method to detect colony counts of *L. monocytogenes* in environmental samples (Nyachuba & Donnelly, 2007)

Methods of Enrichment

Enrichment is used to create a nutrient rich environment for overall growth or selective growth of a desired species. As detection methods used for presence/absence testing of food or environmental samples, enrichment methods are used because when low levels or injured cells are present in a sample, the bacterial target cannot be detected with a general-fast-acting method such as Aerobic Plate counts on Petrifilms™ (Jasson, Jacxsens, Luning, Rajkovic, & Uyttendaele, 2010). The enrichment method is used as a two-step process with either a nutrient dense or semi selective medium made to target a desired pathogen, and a second step using a selective media to further target the desired pathogen (Jasson et al., 2010). The two-step process usually starts by submerging a food or environmental sample into nutrient rich media for about 24 hr to allow growth and repair of target bacteria, and the second step is to use the selective media to suppress any unwanted background flora and only target the desired pathogen during an additional 24 hr enrichment period (Jasson et al., 2010). This method is used during food testing for detecting pathogens including *Salmonella*, *Campylobacter*, *L. monocytogenes,* and *E. coli* (Jasson et al., 2010). For *L. monocytogenes* detection, the U.S Food and Drug Administration (FDA) and United States Department of Agriculture Food Safety Inspection Services (USDA/FSIS) specify methods of enrichment for food producers and third-party laboratories to use when monitoring their facilities.

The FDA method specifies use of Buffered *Listeria* Enrichment Broth Base (BLEB), which is a single enrichment step using a non-selective nutrient media for an initial four-hour incubation period, followed by addition of a cocktail of antibiotics to

make the medium selective for *L. monocytogenes* detection (U.S FDA, 2017b). For environmental sponge samples, the USDA-FSIS Microbiology Laboratory Guidebook (MLG) (USDA/FSIS, 2017a) suggests enrichment of each sponge in 225+/- 5ml of UVM broth with incubation at 30°C for 20-26 hr. Transfer of 0.1ml of UVM enrichment to either 10ml of Fraser broth, with incubation at 35^0C for 26 hr, or MOPS-BLEB broth, with incubation at 25°C for 18-26 hr is conducted. UVM and Fraser broth enrichments are streaked onto MOX plates and incubated for 26 hr at 35°C. Post incubation, the MOX plate is interpreted and further processed depending on absence or presence of growth within 26hr. A study by Pritchard, (1999) recommends use of the dual primary enrichment technique to increase the sensitivity of *Listeria* species detection while decreasing the steps in testing procedures. Two primary enrichment broth media, UVM and LRB, are each used for primary enrichment. Following enrichment, aliquots from each broth are combined into a single secondary enrichment broth (Fraser broth), incubated and confirmed on MOX agar (Pritchard & Donnelly, 1999). This method increased the detection rate of 83% when using a single secondary enrichment method to 93.8% with the secondary enrichment step (Pritchard & Donnelly, 1999).

Methods of Enumeration

Enumeration is a method used to quantitatively identify viable cells in a sample or diluted inoculum (Jasson et al., 2010). This method is commonly used as more of a non-selective, preliminary approach to an investigative experiment or testing. Enumeration can be done by plating onto Petrifilm^{TM} with dehydrated media, or onto a

petri dish with nutritional agar. When counting on Petrifilm™, a readable plate for identifying a target dilution of cells is between 15-150 CFU/ml and when counting a readable plate on petri-dish of agar the target dilution of cells should be around 25-250 cfu/ml. For *Listeria* enumeration 3M™ Petrifilm™ for aerobic bacteria was used, by dispensing 1ml of diluted sample onto the dehydrated medium and incubation for 30-37C° 24-48hr.

Alternative Methods

Rapid Detection Methods

Along with the conventional methods, there are several alternative methods used for rapid detection and identification of *L. monocytogenes*. These methodologies include Polymerase Chain Reaction screening (PCR), molecular subtyping, serotyping, and Ribotyping. For rapid detection of *L. monocytogenes* in a farm or food environmental sample, PCR is a reliable tool (Gwida & Al-Ashmawy, 2014). The method uses DNA polymerase to amplify the specific DNA fragment of the detected organism through a series of heating and cooling steps (Gwida & Al-Ashmawy, 2014). This method may have issues with accurate detection in the case of low level contamination, especially when there is too much background flora in the sample, but there are now PCR assays made for each popular pathogen to target specific serotypes to improve detection rates (Gwida & Al-Ashmawy, 2014). The FDA and FSIS have similar guidance for using PCR when detecting *L. monocytogenes* in food and environmental samples. According to the (USDA/FSIS, 2017b) samples must be prepared in a secondary enrichment and plated from a primary enrichment. Once isolates are obtained, the BAX PCR assay can be used alongside MOX agar. PCR guidelines specified in the FDA BAM (U.S FDA, 2017a) suggest preparing isolates in Brain Heart Infusion medium, and running samples with control strains, such as *L. monocytogenes* ATCC: 19115. Molecular subtyping and serotyping are ways to differentiate between different species of *Listeria*. There are 13 serotype classifications for *L. monocytogenes*, with three serotypes (1/2a, 1/2b, and 4b) causing 95% of human infection (Tompkin, 2002)

Automated ribotyping has become a popular subtyping method because of its ease of use and reproducibility. The method analyzes restriction enzyme digests of total genomic DNA that is hybridized with a ribosomal (rRNA) probe. The resulting fingerprint pattern is representative of the number and sequence diversity of the rRNA operons.(Nadon, Woodward, Young, Rodgers, & Wiedmann, 2001). It is able to not only differentiate *L. monocytogenes* from other *Listeria* species but can do it with a large data base to trace similar ribotyped patterns and link to already existing strains (Nadon et al., 2001).

Methods used for this Study

Two standard enumeration methods were used for this study, which included 3M™ Aerobic Plate Count Petrifilm™ and 3M™ *Listeria* Environmental plates, to qualitatively recover high concentrations of *Listeria* species from wooden surfaces. To recover low concentration levels of *Listeria* species on wooden surfaces, four standard enrichment methods, modified FDA, primary FDA, dual enrichment, and modified USDA are used. When measuring performance of environmental sampling tools in a real-time farm setting, two of the four enrichment methods were chosen based on their performance from the controlled lab experiments. Those two enrichment methods were modified USDA enrichment and Dual enrichment which was further processed by screening for *Listeria* species using a Genus: *Listeria* species PCR assay and DuPont Qualicon BAX Q7 system. If any *Listeria* species were found and confirmed by plating to CHROMagar™ *Listeria* base, the confirmed samples were then processed through ribotyping with the Riboprinter® Microbial Characterization System by Hygiena.

Level of Risk for Artisan Cheese Ripened on Wooden Shelves

To understand the risk of manufacturing artisan cheese, a microbial risk assessment can be done to measure the level of risk for hazardous microorganisms that could contaminate finished product. A risk assessment is a scientific method used to identify exposure, characterizations and preventive measures for controlling microbiological hazards during production (Choi et a., 2016). Choi et al., (2016) categorized cheese by their moisture content, since bacterial pathogens survive well in high moisture environments. Low moisture cheese are known to have less than 50% moisture and include cheeses such as the semi-soft cheeses Stilton, Roquefort, Gorgonzola, Limburger, Gouda, and Edam. Some hard cheeses (35-45% moisture) include Cheddar, Emmental, Gruyere and lastly very hard cheeses (13-34% moisture) including Parmesan, Asiago old, Romano, Grana, etc. have little to no risk of microbial growth during their aging period. Table (1) shows each artisan cheese that is ripened on wooden shelves and the level of risk for each pathogen of concern, based on information provided by Choi et al., (2016).

Table 1a. Correlation and level of risk for pathogenic contamination (Choi et al., 2016)

Cheese	Moisture	Pathogens of Concern	Level of Risk
Cheddar Cheese	37% to 42%	*E. coli*	High
Blue cheese	39$ to 50%	*L. monocytogenes*	High
Camembert	>55%	*E. coli*, *L. monocytogenes*	High
Soft-ripened raw milk cheeses	>50%	*L. monocytogenes*	High
Soft-ripened pasteurized milk cheese	>50%	*L. monocytogenes*	Low

Other than moisture, the materials and process used to make each cheese are also significant factors when measuring the risk of each cheese. Raw milk, cheese washing brushes, and wooden shelves used for aging can all be vehicles of pathogenic bacteria in artisan cheese manufacturing facilities. Raw milk is a significant vehicle for *L. monocytogenes* from mastitic cows, animal shedding, and improperly fermented silage (Kozak, Balmer, Byrne, & Fisher, 1996). Kozak et al., (1996) states that although only 3-4% of raw milk used in cheese making is known to be contaminated with *Listeria* species, there is still a chance of *Listeria* species contamination post pasteurization. Pasteurization is a critical control point where the temperature and time of heat treat raw milk must be consistent at all times. Most cheese related foodborne illnesses are linked back to improper pasteurization of raw milk (Choi et al., 2016).

This is a good reason why cheese makers, especially small-scale artisan cheese makers, need to ensure they are following their good manufacturing practices, where proper cleaning and hygienic protocols are stringently followed and monitored to ensure all finished product is safe from potential contamination.

Implications of this Study

Wood will always be questionable as a food contact surface because of its porosity and rough surface which allows for a high risk of pathogen survival, biofilm formation, and cross contamination if cheesemakers are consistent with application of their SSOPs and environmental monitoring programs. There are many efforts being made to ensure that wood is safe and can be confidently used for traditional practices of artisan cheese making. A review of the microbial and safety of wood was done by Aviat et.al in 2016. These authors cited 86 studies to prove that wood is indeed a suitable food contact surface and packaging material, and not only for the functionality of aging cheese or containing product, but wood also has an appeal to consumers of “organic”, “crafted”, and “natural” products. The review also stated that wood benefits cheese production by imparting quality, safety, and tradition.

One big advantage of using wooden shelves for aging cheese is the formation of biofilms during the cheese aging period. This biofilm is formed by naturally occurring bacteria from the milk and starter cultures which usually include yeast and lactic acid bacteria and known as LAB (Lactic Acid Biofilms) (Galinari, Nóbrega, Andrade, & Luces Fortes Ferreira, 2014). The LAB can be beneficial in developing the desired quality and character of the finished cheese products. Although biofilms may be encouraged, cheesemakers have to monitor what kind of bacteria are forming the biofilms, as some pathogenic bacteria such as *Staphylococcus,* and *E. coli* may develop biofilms from employee handling, environmental cross contamination, or from the milk used in cheesemaking (Galinari et al., 2014).

A biofilm is defined as a community of surfaced microbial cells that protect themselves in an self-made extracellular polymeric substance matrix (Donlan, 2002). This extracellular polymeric substance is mostly comprised of polysaccharide material, and provides an ideal environment for the cells to attach to a surface and create a community where all bacteria communicate to survive (Donlan, 2002). These biofilms tend to be hard to remove from surfaces, especially wooden surfaces that have many pores allowing biofilm attachment, requiring all cleaning and sanitation protocols to include intensive procedures involving application of chemical solutions, heat treatments, and drying to destroy the biofilms existing within the shelves. There are certain sanitizers that have been found to effectively work against biofilms containing *L. monocytogenes* communities, including peroxyacetic acid sanitizers and rotation of sanitizing agents to prevent persisting strains of *L. monocytogenes* from becoming sanitizer resistant (Pan et al., 2006). Certain characteristics of environmental surfaces promote successful biofilm attachment, including rough and hydrophobic surfaces, and the ability to hold water, temperature and access to nutrients (Donlan, 2002). But what allows the pathogen such as *L. monocytogenes* to first attach to the wooden shelves to then allow for further formation of biofilms? A study was done by authors Bae, Seo, Zhang, and Wang (2013) and identified what genes caused *Listeria* species such as *L. monocytogenes* to attach to plant material. The study identified a LCP gene which was accompanied by a putative CBD gene within the *L. monocytogenes* genomic map, which allows for cell attachment to the plant's cell wall composed of cellulose (Bae, Seo, Zhang, and Wang 2013). This binding is important to understand not for only vegetables but even for wooden material, because it can be linked to the start of contamination and biofilm formation from attachment to

the cellulose within the material of the shelves used for cheese aging. These wooden shelves have all of these characteristics, with the rough porous surface, ability to contain water, temperature, and a supply of nutrients from the cheese. If the cheese placed on the wooden board was contaminated with *L. monocytogenes*, these cells would have the opportunity to form a strong biofilm to multiply and survive, because cheese is ripened on these wooden boards for a long period of time. With stringent cleaning and sanitation procedures as well as a post environmental sampling of the sanitized wooden boards, there should either be no detection of biofilms.

Raw milk cheese made without a curd cooking process can be especially risky when using wooden shelves because there is no heat treatment to kill off any environmental pathogens from the milk or cheesemaking process. It has been reported that the reoccurring rate of pathogens in raw milk is between 3-6.5%, and *L. monocytogenes* is recognized as an important source of post processing contamination (Ibba et al., 2013) and can with stand low temperatures, and high NaCl concentrations in environmental niches (Ibba et al., 2013). These niches do encourage the biofilm formation of pathogenic bacteria and result in a more difficulty in detection during environmental sampling. Unfortunately, the niches in a wooden shelve are within the pores of the shelves and cannot be taken apart to be decontaminated and may impact proper monitoring or detection of *L. monocytogenes*., SSOPs must therefore assure full decontamination of potential pathogenic bacteria.

Environmental niches, poor environmental monitoring programs, and poor sanitation protocols can all lead to contamination of finished product and food-borne illness outbreaks. In 2011 the Center for Disease Control and Prevention (CDC),

provided an accurate census of foodborne outbreaks in the US, they reported that 48 million people become ill, 128,000 people are hospitalized, and 3,000 deaths are caused annually from foodborne outbreaks (Scallan et al., 2011). In order to reduce outbreaks of listeriosis from artisan cheeses, 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.

This collaborative study experimented with four environmental surfaces, Marie Limoges PhD was responsible for collection of data from three surfaces of plastic, dairy brick, and stainless steel, while I collected data from wooden surfaces. These data were independently analyzed, then combined for final statistical analysis. I contributed to writing on the portions of the manuscript most relevant to methods and regulations, which applied to the wooden surfaces.

Chapter 2: Comparative Recovery of *Listeria* spp. From Dairy Environmental Surfaces Using 3M™ And World Bioproducts© Environmental Swabs and Standard Enrichment and Enumeration Methods

Authors:

Marie Limoges[*1], Gina Frontino[1], Catherine Donnelly[1]

[1]Department of Nutrition and Food Sciences, University of Vermont, Carrigan Wing, 109 Carrigan Drive, Burlington, Vermont 05405

[*]Author correspondence to mlimoges@uvm.edu, 802-578-5644

Keywords: *Listeria*, recovery, environmental sampling program, environmental sponges, swabs, efficacy, FSMA

Formatted for Submission to the Journal of Food Protection

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, 2017; 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 (FDA 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 (FDA 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 (FDA CFR, 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; FDA CFR, 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, 2017b; 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, 2017a) 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, 2017a). 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, 2017a) , 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.

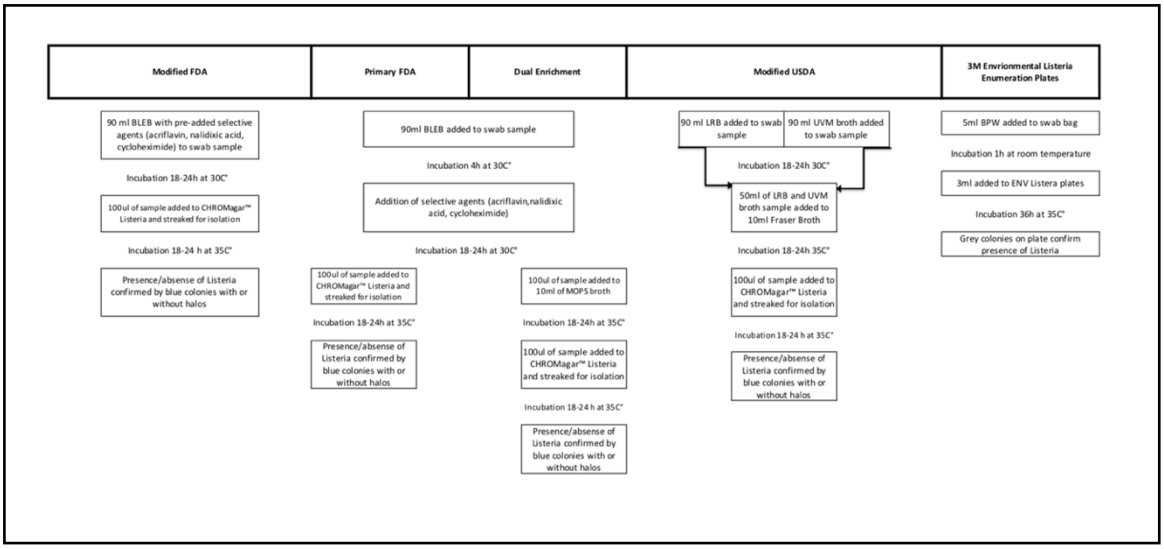

FIG 1: Enrichment Methods against 3M™ ELP Petrifilm™

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.

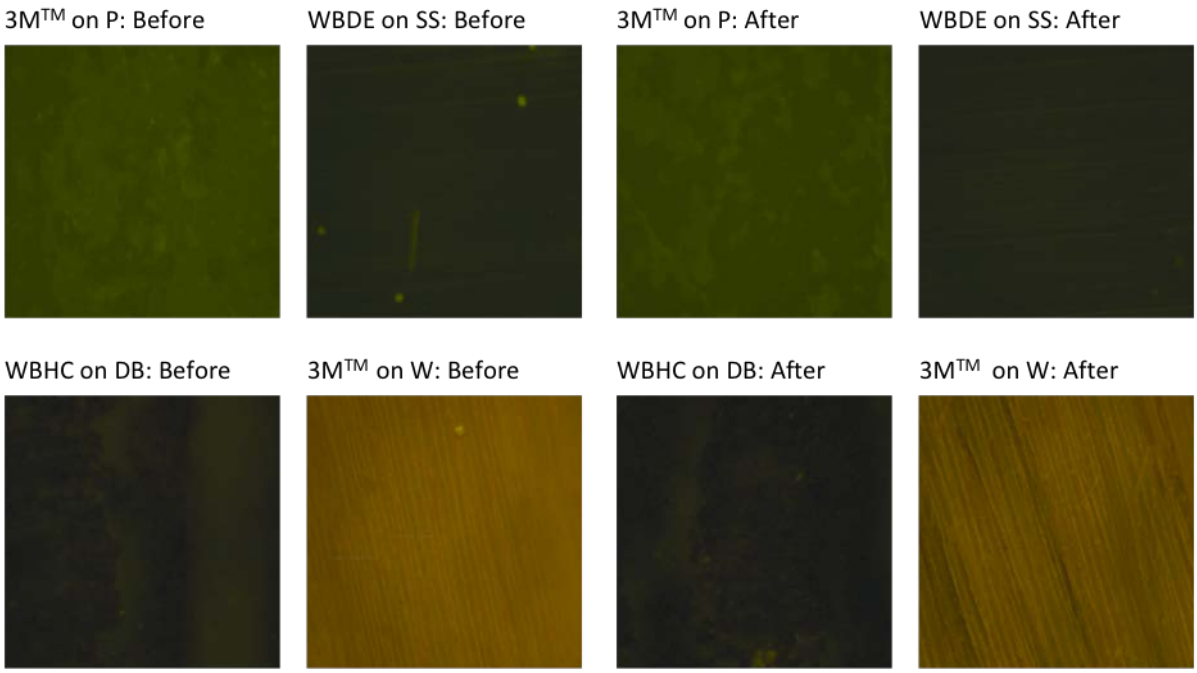

FIG 2: Comparison Using MI Between All Swab Formats at 11.5x Magnification. Left group: before swabbing (top left: 3MTM and P (plastic); top right: WBDE and SS (stainless steel; bottom left: WBHC and DB (diary brick); bottom right: 3MTM and W (wood)). Right group: after swabbing top left: 3MTM from P (plastic); top right: WBDE from SS (stainless steel); bottom left: WBHC from DB (dairy brick); bottom right: 3MTM and W (wood). Other data not shown.

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 3) 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).

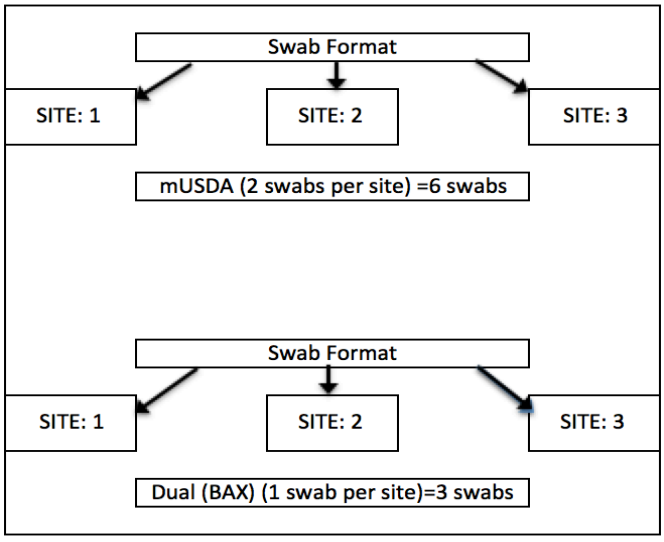

FIG 3: Farm Site-Environmental Sampling Plan

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^{TM} (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^{TM} (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 2). 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^{TM}, 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^{TM}) for all surfaces were also observed (Table 10). Of swabs tested, 3M^{TM} 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^{TM} 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.

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 et al., 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/cm^2	**0.1 CFU/cm^2**	**1 CFU/cm^2**	**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^{TM}	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^{TM} 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^{TM} EL Plate= 3M^{TM} 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 Plate

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

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 (FDA 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 (U.S. FDA, 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.

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