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INVESTIGATING THE PREVALENCE AND CONTROL OF *LISTERIA MONOCYTOGENES* IN FOOD FACILITIES

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Small and very small food facilities in the ready-to-eat food industry face difficulties complying with the Food Safety Modernization Act-Preventive Controls for Human Food rule (FSMA-PCHF). This regulation highlights the need for sanitation to control environmental pathogens like *Listeria monocytogenes*. The main goal of this project was to investigate the prevalence and control of *Listeria monocytogenes* in food facilities. This study provides technical assistance to facilities to comply with the PCHF rule and addresses sanitation alternatives for food contact surfaces like aqueous ozone.

First, the prevalence of *Listeria* spp and *L. monocytogenes* in small and very small food manufacturing facilities in Nebraska was determined. In this study, environmental samples were collected from three participating facilities. Overall, *Listeria* spp were detected in 14 of 266 (5.3%) samples with sites like floors and drains having the highest prevalence. No significant difference in prevalence across all three facilities was observed. *Listeria monocytogenes* was not detected in any of the facilities. This study highlights the importance of management and sanitation of non-food-contact surfaces like drains and floors. Our data was provided to participating facilities to assist in starting their environmental monitoring program and overall, contributing to their compliance with the PCHF rule.

Next, we determined the efficacy of ozonated water for the decontamination of *Listeria* on food contact surfaces. For this study, stainless steel and polypropylene coupons constructed to

10 x 10 cm were conditioned with organic matter made from uncured deli turkey breast and inoculated with *Listeria monocytogenes*. Other experiments were performed with *Listeria innocua* separately. Clean coupons were also included for experiments with *L. innocua*. Inoculated surfaces were exposed to 10 ppm of ozonated water for 15, 30, and 45 seconds. Tap water was included as a treatment. There were no significant differences in reductions attributed to ozonated water compared to tap water washing. However, reductions of *L. innocua* on soiled stainless steel were significantly higher than on clean surfaces($P = 0.01$). Similarly, *L. innocua* reductions were numerally higher on soiled polypropylene though not significantly different from clean coupons. Spraying applications may have influenced bacterial reduction from surfaces by dislodging rather than actual inactivation. In addition, the soil system with deli turkey may have not provided sufficient soil (grease) to reduce the efficacy of ozonated water resulting in similar reductions on both soiled and clean surfaces. Overall, data suggest that cleaning may be effective at reducing transiently attached *Listeria* from FCS.

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1.1. *Listeria monocytogenes*

1.1.1 Microbiology and Survival

Listeria monocytogenes is a gram-positive, non-spore forming bacterium that can inhabit the soil, water sources, livestock, and humans, posing a continuous threat to food safety. This facultative intracellular pathogen can cause severe invasive illness in at-risk human populations (McMullen & Freitag, 2015). The species is subdivided into 13 serovars, and a vast majority of human cases are linked with serovars 4b, 1/2a, and 1/2b. *Listeria* possesses one to five peritrichous flagella which confer swimming and tumbling motility at 28 °C; however, most strains have reduced or no evident motility at higher temperatures, including the human body temperature (37 °C). Its optimum growth temperature ranges from 30 to 37 °C, although growth has been reported from -0.4 to 42 °C. When grown in blood agar, colonies appear weakly hemolytic (Dongyou et al., 2020; International Commission on Microbiological Specifications for Foods (ICMSF), 1996). This pathogen is known for its ability to survive or even replicate under a wide range of environmental stress conditions (Ferreira et al., 2014; Gahan & Hill, 2014). Stress resistance supports the colonization and persistence of *L. monocytogenes* in various niches along the food chain and ultimately contributes to the ability of this bacterium to infect humans (Berrang et al., 2010; Bolocan et al., 2016; Leong et al., 2014; Sleator et al., 2009). The stresses encountered by *L. monocytogenes* in food are a result of the intrinsic properties of the food including acidic pH in fermented foods, osmotic stress by increased salt concentrations, and more contemporary ones like bacteriocins and other food preservatives that inhibit the growth of this bacterium (Albarracín et al., 2011; Johnson et al., 2018; Leroy & De Vuyst, 2004). Extrinsic stress factors are those measures of food preservation that are designed to kill this pathogen at

the processing stage or protect the food during storage. Examples include thermal treatments or alternatives like pulse electric fields, high-pressure processing, low temperatures/refrigeration, etc. (Escajeda et al., 2018; Morris et al., 2007).

The psychrotolerant nature of *L. monocytogenes* is responsible for its relatively frequent detection in refrigerated food products especially refrigerated ready-to-eat (RTE) meat, poultry, and seafood products (Tasara & Stephan, 2006; U.S. Department of Agriculture, 2003). Low temperatures result in decreased metabolic rates, changes in membrane composition, the expression of cold shock proteins (Csps), and the uptake of cryoprotectants (Cordero et al., 2016; Neunlist et al., 2005; Phadtare et al., 1999). These changes maintain the fluidity of the membrane and prevent the formation of a gel-like state that may result in leakage. They also contribute to stabilizing the conformation of nucleic acids and prevents degradation thus facilitating replication, transcription, and translation of proteins at low temperatures (Barria et al., 2013; Beales, 2004; Lee et al., 2012). Under such conditions, osmolytes like glycine, betaine, carnitine, gamma butyrobetaine, proline betaine, and 3- dimethylsulphoniopropionate are imported as cryoprotectants (Chan et al., 2007). Osmolytes such as carnitine and glycine betaine (also known as compatible solutes) are also accumulated when *L. monocytogenes* is exposed to elevated concentrations of salt. The osmolytes in this case reduce the osmotic pressure and water loss hence keeping cell turgor pressure under control (Duché et al., 2002). Besides the turgor pressure, osmolytes contribute to stabilizing enzymes' structure and function during stress (Lippert & Galinski, 1992). Its resistance to high osmolarity has been demonstrated by growth up to 13% NaCl(Liu et al., 2005; Shabala et al., 2008).

This pathogen may also face oxidative stress from sanitizers. Under these conditions, cells encounter high concentrations of oxygen radicals (Suo et al., 2014). These radicals disturb

the normal redox state of cells leading to cell death due to the oxidative damage of proteins, lipids, and nucleic acids. Bacteria use reduction pathways to repair the damage of susceptible amino acids induced by reactive oxygen species or reactive chlorine species. These compounds activate enzymes such as superoxide dismutases, catalases, peroxidases, and efflux pumps to counteract oxidative stress (Archambaud et al., 2006; Dröge, 2003). Exposure to a stress factor can provide cross adaptation to subsequent exposure to other stresses (Begley et al., 2002; Bergholz et al., 2012). For example, incubation of *L. monocytogenes* at low temperatures enhances its resistance to high salt concentration (Schmid et al., 2009). Additionally, osmotic stress can lead to cross-protection against high temperatures, ethanol, alkalinity, acidity, and oxidative stress (Melo et al., 2015).

Biofilms are one of the main sources of repeated *Listeria* food contamination (Chmielewski & Frank, 2003; Colagiorgi et al., 2017; Giaouris et al., 2015). Biofilms are created by microorganisms adhering to surfaces and growing as sessile communities. They are the predominant mode of microbial development in nature. Materials used in the food processing environment such as stainless steel, polypropylene, glass, or rubber can support *L. monocytogenes* colonization and biofilm formation (Beresford et al., 2001; Chavant et al., 2004). The extracellular polymeric matrix gives extra protection from harsh environmental conditions such as desiccation, nutrient deprivation, or disinfection when this pathogen is organized in a biofilm (Bridier et al., 2011; Esbelin et al., 2018).

Overall, *L. monocytogenes* uses diverse mechanisms to survive various stress conditions encountered in food matrices and the environment hence it is important to understand the microbiology of this pathogen to develop more efficient methods to reduce its occurrence in food and the food environment.

1.1.2. Public Health Significance of *Listeria monocytogenes*

Most *L. monocyotogenes* infections are commonly associated with foodborne outbreaks involving cheese, deli meats, and produce (McMullen & Freitag, 2015). The ability of *Listeria* to survive a variety of environmental conditions may contribute to the diversity of clinical manifestations associated with the organism. The manifestation of infection may depend on the patient's predisposition or potentially on strain-specific bacterial factors that influence the progression of the disease. Gastroenteritis originally thought to be silent may be apparent within 48 hours of exposure (Schlech, 1997). Symptoms of gastroenteritis are like those caused by enteric pathogens and may include nausea, watery or bloody diarrhea, abdominal pain, and fever. The occurrence of early symptoms is dependent on the quantity of ingested inoculum as suggested by studies in non-human primate model. In healthy individuals, the disease is often self-limiting (Farber & Peterkin, 1991; Schlech, 1997). A majority of the diagnosed cases of listeriosis are invasive. This is when the bacterium spreads from the GI tract into the bloodstream by breaching the epithelial barrier of the intestines through the expression of different virulence genes (*inlAB* internalization locus, *Listeria* pathogenicity island-1 (*LIPI-1*), and *hpt* intracellular growth locus, respectively) (Centers for Disease Control and Prevention (CDC), 2018; Vázquez-Boland et al., 2001). This invasive form of the disease is common in individuals with conditions that predispose them to illness. Some of these conditions are neoplastic disease, immuno-suppression, pregnancy, extremes of age, diabetes mellitus, alcoholism, cardiovascular and renal collagen diseases, and hemodialysis failure. Invasive listeriosis causes meningitis, septicemia, primary bacteremia, endocarditis, central nervous system infections, influenza-like illness, and conjunctivitis. Sepsis in pregnant women may lead to abortion, stillbirth, premature birth, or septicemia in newborns (Farber & Peterkin, 1991). An estimated 1,600 people are sickened with

listeriosis annually in the U.S. with 90% of these cases requiring hospitalization (Hoffman et al., 2015). It is also responsible for an annual average of 282 congenital illnesses due to women getting infected during pregnancy. While women may recover, congenital illness may reduce the chances of survival of the fetus.

Listeriosis accounts for 19% of the total deaths caused by a major foodborne pathogen (Hoffman et al., 2015; Scallan et al., 2011). It is one of the top five pathogens responsible for 90% of the economic burden of foodborne pathogens and is one of the top 2 leading causes of deaths on a per case burden, costing $1.8 million/case and surpassed only by *Vibrio vulnificus*. In a typical year, *L. monocytogenes* imposes an estimated $2.8 billion ($227 million to $7.6 billion range) in total economic burden due to medical costs, productivity losses, and cost of death (Batz et al., 2011; Hoffman et al., 2015; Hoffmann et al., 2012; Scallan et al., 2011). Because of its public health significance and economic impact, the control of this pathogen in industry is critical to ensure consumer safety particularly in foods that are subject to little or no lethality treatment like ready-to-eat foods.

1.1.3. Ready-to-Eat foods (RTE foods)

Ready-to-eat foods are defined as any food that is normally eaten in its raw state or any other food, including processed food, for which it is reasonably foreseeable that the food will be eaten without further processing that would significantly minimize biological hazards (21 CFR 117.3) (U.S. Food and Drug Administration, 2020). The United States Department of Agriculture, Food Safety, and Inspection Service (USDA-FSIS) defines RTE meat and poultry products as products that are safe to eat without additional preparation, although they may receive additional preparation (for example, reheating) for palatability or aesthetics, epicurean, gastronomic, or culinary purposes. This category may include fully cooked RTE products that do

not need further preparation by the consumer, frozen meat and poultry products which can be eaten as they are or reheated for palatability, and fresh or frozen entrees with fully cooked meat or poultry portions (designed to be reheated) combined with fully cooked sauces, vegetables, pasta, or other ingredients. Some examples of RTE products are hot dogs, luncheon meats, cold cuts, fermented or dry sausage, and other deli-style meat and poultry (USDA-FSIS, 2019). Because RTE foods may not receive additional treatments from consumers to eliminate any residual microorganisms, their microbiological safety is critical to minimize the risk of foodborne illness or outbreaks particularly for foods that are refrigerated for extended durations. (Ivy et al., 2012).

1.1.4. Outbreaks of Listeriosis in the United States

Between 1998 to 2008, regulatory changes have resulted in a decrease in the number of outbreaks involving RTE meat and poultry products (Cartwright et al., 2013; Luchansky et al., 2017). However, there has been no marked decrease in outbreaks involving dairy products (Cartwright et al., 2013). Additionally, the U.S. has experienced outbreaks involving foods that are considered ‘low risk’ or ‘moderate risk’ including ice cream (Food and Drugs Administration, 2003). Table 1.1 summarizes listeriosis outbreaks that have been reported in the U.S. since 2012 (CDC, 2021a).

Table 1. 1. Outbreaks of Listeriosis in the U.S. from 2012-2021 (CDC, 2021a)

Year	Food vehicle	States	Case Count	Hospitalizations	Deaths
2021	Queso Fresco	4	13	12	1
2021	Deli meats	4	12	12	1
2020	Enoki mushrooms	17	36	31	4
2019	Hard-boiled eggs	5	8	5	1
2019	Unidentified food vehicle	13	24	22	2
2019	Deli-sliced meats and cheeses	5	10	10	1
2018	Asian style pork patties	4	4	4	0
2018	Deli ham	2	4	4	1
2017	Raw milk cheese	4	8	8	2
2016	Frozen vegetables	4	9	9	3
2016	Raw milk	2	2	2	1
2016	Packaged salads	9	19	19	1
2015	Soft cheeses	10	30	28	3
2010-2015	Ice cream	4	10	10	3
2015	Caramel apples	12	35	34	7
2015	Mung bean sprouts	2	5	5	2
2014	Fresh curd cheese	4	5	4	1
2014	Cheese	2	8	7	1
2012	Ricotta Salata cheese	4	22	20	4
2012	Cantaloupe	28	147	143	33

1.1.5. Prevalence of *Listeria monocyotogenes* in RTE Foods

Initial causes of listeriosis in the U.S in the 1990s were associated with the consumption of deli meats and hot dogs (Gillespie et al., 2006; Goulet et al., 2012; U.S. Centers for Disease Control and Prevention, 2021b). After the initiation of policy changes such as defining objective levels of pathogen growth and environmental testing of establishments, a reduction in prevalence was achieved. In recent years, listeriosis outbreaks in the U.S. have more commonly been associated with contaminated dairy and raw produce especially packaged salads that are an emerging concern of *L. monocytogenes* contamination (Buchanan et al., 2017; Gottlieb et al., 2006; Mead et al., 2006).

The estimation of the prevalence of *L. monocytogenes* in various foods may be useful for many groups. It can be used by policymakers to inform testing parameters for surveillance. Additionally, these estimates can also be used by medical professionals when consulting with immunocompromised, elderly, or pregnant individuals regarding the risk of infection and by health units to educate health inspectors and the general population about high-risk foods. Finally, estimates of prevalence can be used to inform risk assessments for *Listeria monocytogenes* contamination (Churchill et al., 2019).

A review of a hundred studies (included studies between 1980 to 2017) worldwide with a sample size restriction of ≥100 by Churchill et al., (2019) on the prevalence of *L. monocytogenes* in high-risk food estimated the overall *L. monocytogenes* prevalence in deli meat at 2.9% (95% confidence interval [CI] 2.3 to 3.6%, in soft cheese at 2.4% (95% CI, 1.6 to 3.6%) and in packaged salads at 2.0% (95% CI, 1.2 to 3.1%). A substantial difference in prevalence among studies used in this review was suggested to be caused by unmeasured factors such as cross-contamination, geographical location, sanitation practices, testing methods and, variations in temperature during storage and transportation. Policies also account for variation in the prevalence of *L. monocytogenes* (Luchansky et al., 2017). For example, a 14 to 24 months survey before implementation of regulations for control of *Listeria* in food revealed a prevalence of 0.74 to 2.36% in salads, 0.17 to 1.42% in cheeses, and 2.36% in processed meats (Gombas et al., (2003). Conversely, Luchansky et al., (2017) reported much lower levels after regulatory agencies made changes in regulations and industry had taken measures to control for *Listeria* in food products. In this survey the prevalence of *Listeria* was 0.18 to 0.25% in deli meat, 0.0% to 0.16% in dairy and 0.28 to 0.85% in salads. Hence the prevalence of *L. monocytogenes* may vary from one food category to another based on several factors and is not consistent across studies.

1.1.6. Regulatory Framework for RTE foods

The U.S. enforces a zero-tolerance policy for *L. monocytogenes* in RTE foods. Zero-tolerance refers to regulatory policies where the target microorganism must be absent from a food sample given a specific sampling plan. *i.e*., absence of *L. monocytogenes* in 25-gram samples (*i.e*., fewer than 1 cell in 25 g, or less than 0.04 cells in 1 g) (National Advisory Committee on Microbiological Criteria for Foods, 2010). As such, food products that are contaminated with *L. monocytogenes* are considered adulterated. Besides the zero-tolerance policy, food business operators have other regulatory requirements to follow based on the federal agencies that oversee the products manufactured in their establishment. For example, facilities under the jurisdiction of the USDA-FSIS are required to follow the Listeria Rule. This rule requires RTE meat processors to adopt one of the three designated "Alternatives" to control *L. monocytogenes* on their products. In Alternative 1, processors are required to use both post-lethality treatments that reduce or eliminates *L. monocytogenes* and an antimicrobial agent in the product formulation or process that suppresses or limits the growth of *L. monocytogenes* throughout the product shelf-life. In the case of Alternative 2, the processor must use either a post-lethality treatment that reduces or eliminates *L. monocytogenes* or an antimicrobial agent or process that suppresses or limits its growth throughout the product's shelf life. Finally, in Alternative 3, processors rely only on sanitation measures to control *L. monocytogenes.* All post-lethality treatments and antimicrobials are required to be used at permissible levels and must be validated for the effectiveness in limiting the growth of *L. monocytogenes* to 1 to 2 $\log_{10}$ through the duration of the product shelf-life (USDA-FSIS, 2014). The FDA, on the other hand, requires domestic and foreign food facilities that must register with section 415 of the Food, Drug, and Cosmetic Act to comply with requirements for risk-based preventive controls mandated by the

Food Safety Modernization Act (FSMA; 21 CFR 117c) as well as the Current Good Manufacturing Practices (CGMP). These risk-based preventive controls include sanitation preventive controls that are aimed at maintaining the facility in a sanitary condition to minimize or prevent hazards such as environmental pathogens including *L. monocytogenes*. It also requires that sanitation be verified through environmental monitoring. CGMPs address topics like such as personnel, buildings and facilities, equipment and utensils, production and process controls, and warehousing and distribution (FDA, 2020a).

Overall, these requirements underscore the use of sanitation to control *L. monocytogenes* which is an environmental pathogen.

1.2. Sanitation in the Food Industry

Sanitation establishes the basic hygienic conditions needed to produce safe and wholesome food. Potentially hazardous contamination is introduced into the processing environment without an effective sanitation program (Food Safety Preventive Controls Alliance (FSPCA), 2016; Marriott & Gravani, 2006). Maintaining a clean and sanitary plant is essential in building and executing an effective food safety program and facilities must be vigilant in combatting bacterial contamination and cross-contamination to protect their brand and reputation, protect consumer health, and meet regulatory requirements (Schug, 2018).

Sanitation is the application of practices and procedures to provide wholesome food processed, prepared, merchandised, and sold in a clean environment by healthy workers; to prevent contamination with microorganisms that cause foodborne illness, and to minimize the proliferation of food spoilage microorganisms (Marriott & Gravani, 2006). Poor hygienic and sanitary practices can contribute to outbreaks of foodborne illnesses and cause injury. Additionally, it can contribute to the loss of quality through spoilage and lead to regulatory

action against a company. But the importance of sanitation goes beyond regulatory action. It is a significant program that impacts allergen, microbiological, pest, and/or safety issues within the plant (Reeve, 2014).

Microbiological issues represent a significant reason for food safety failures and are commonly introduced from food facility environment, employees, ingredients/raw materials, or equipment. An effective sanitation program can mitigate risks in these areas (Reeve, 2014). For example, sanitation allows companies to control spoilage organisms which are often the cause of off-condition products. Off-condition products may result in litigation in cases where consumer thinks they may get sick. An effective sanitation program allows companies to avoid such situations. Hence, improved product shelf-life and quality can be achieved through an effective sanitation program. (Marriott & Gravani, 2006). Environmental pathogens like *Salmonella* spp and *Listeria* are major food safety hazards for many RTE foods that are exposed to the processing environment post-lethality. Sanitary facilities are important to prevent cross-contamination by these biological hazards (FSPCA, 2016). Environmental monitoring of these pathogens has recently been gaining greater focus from food companies and regulatory agencies (Reeve, 2014).

Sanitation is a key component in some food safety regulations. For example, sanitation has been part of the CGMP (21 CFR 117.b) (Anonymous, 2021). This regulation generally requires that personnel, fixtures, and food facility be maintained in a sanitary manner to prevent food safety issues. It also requires that sanitary operations adequately protect against cross-contamination or allergen cross-contact (Anonymous, 2021; Reeve, 2014). A well-designed sanitation program is required for food safety management programs like Hazard Analysis and Critical Control Points (HACCP). HACCP is required for some sectors of the food industry like

juice, seafood, and meat industries. Without sanitation programs acting as pre-requisites, such programs will not be effective (Reeve, 2014). The FSMA (21 CFR 117c) (Anonymous, 2021) is the most recent and most significant change for the food industry. Under FSMA, sanitation is required as a preventive control to ensure that the environment is maintained in a sanitary manner to prevent the cross-contamination of environmental pathogens such as *Listeria monocytogenes*, *Salmonella* spp., *E. coli*, etc. This regulation requires sanitation programs to have specific monitoring, verification, validation, and corrective measures in place. Because FDA inspectors inspect records to prove that these activities occurred it is important to document the written procedure of sanitation in the food safety plan (FSPCA, 2016; Reeve, 2014).

1.3. Concluding Remarks

Because of the public health significance of *L. monocytogenes*, U.S. federal agencies have implemented a regulatory framework and require food business operators under their respective jurisdictions to implement and document control measures. Some regulations like FSMA-PCHF and the *Listeria* control rule are emphatic on the use of sanitation for the control of *Listeria* that is an environmental pathogen that can easily contaminate food surfaces and food products.

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CHAPTER 2. PREVALENCE AND MAPPING OF *LISTERIA* SPP AND *LISTERIA MONOCYTOGENES* IN SMALL AND VERY SMALL FOOD MANUFACTURING FACILITIES IN NEBRASKA

Abstract

Under the FSMA-PCHF rule, environmental monitoring of *L. monocytogenes* is required especially for RTE foods if post lethality contamination is a risk. Meeting this requirement presents more obstacles for small and very small food businesses due to limited resources and a lack of in-house expertise. This study was aimed at determining the prevalence of environmental *Listeria* spp/*L. monocyotogenes* in small and very small food manufacturing facilities in Nebraska. The study was carried out in three food processing facilities (A, B, and C) and included two RTE and one non-RTE frozen food company. In each facility, 25 to 30 sites representing zones 1 to 4 were identified, mapped, and swabbed. Each facility was visited three times and samples were collected during production, shortly before sanitation. Presumptive *Listeria* spp/*L. monocytogenes* positives were detected using the 3M™ Petrifilm Environmental *Listeria* Plates and the 3M™ Molecular Detection Assay *Listeria* according to the manufacturer's instructions. Overall, *Listeria* spp were detected in 14 of 266 (5.3%) samples. *Listeria* spp were detected in all facilities; A (4.4%; 4/92), B (5.9%; 5/85) and C (5.6%; 5/89). No significant difference in prevalence across all three facilities was observed. In addition, *L. monocytogenes* was not detected in any of the facilities. The prevalence of *Listeria* spp was higher when 3M™ Petrifilm Environmental *Listeria* Plate (4.9%; 13 of 266) was used for sample analysis than when 3M™ Molecular Detection Assay *Listeria* was used (0.3%; 1 of 266). Non-food contact surfaces like drains and floors had the highest frequency of *Listeria* spp positive samples. Our data support the importance of management and sanitation of non-food contact

surfaces like drains and floors. This study also provides data that will enable participating facilities to start an environmental monitoring program and overall, contribute to their compliance with FSMA-PCHF rules.

2.1. Introduction

Listeria monocytogenes is a foodborne pathogen that causes febrile gastroenteritis with typical food poisoning symptoms like abdominal cramps, nausea, and diarrhea. In severe cases, the infection may progress to an invasive illness where *L. monocytogenes* breaches the epithelial barrier of the intestinal tract causing septicemia, meningitis, or other infections of the central nervous system in susceptible individuals (European Food Safety Authority, 2014). This pathogen is ubiquitous and can easily enter the food processing environment through ingredients, workers, and vehicles, contaminating food and food contact surfaces (FCS) hence, making it a relevant environmental pathogen in the food chain (Malley et al., 2015). Most recalls and outbreaks of foodborne listeriosis have often been traced back to contamination sources in the environment and equipment of processing facilities (Ferreira et al., 2014; Malley et al., 2015; Zoellner et al., 2018). For example, in 2018, a deli ham-borne outbreak caused 4 cases of listeriosis which resulted in one death. An environmental assessment at the establishment by the United States Department of Agriculture, Food Safety, and Inspection Service (USDA-FSIS) yielded several factors potentially contributing to contamination and specifically related to the environment of the deli ham processing facility. Another outbreak in 2015 involving dairy products led to ten cases of listeriosis and resulted in three deaths across four states. Like most outbreaks of listeriosis, environmental sample analysis led to the detection of a matching *L. monocytogenes* strain on non-food contact surfaces (NFCS) around the processing room and equipment (FDA, 2015).

Cross-contamination of food products from the environment is a major concern in the RTE food industry, especially for foods that do not undergo any post-lethality treatment (fresh-cut fruits, sandwiches, raw produce including salads and, wraps) or that are exposed to the environment post lethality. For example, Muhterem-Uyar et al. (2015) conducted an extensive sampling program in meat plants and reported that some food processing environments previously determined as uncontaminated were contaminated at least once hence showing the existence of a consistent risk for cross-contamination. In another study, there was evidence of cross-contamination between the processing environment and food through indistinguishable *L. monocytogenes* pulsotypes found in both the environmental and food samples (Leong et al., 2017). These reports demonstrate that food products may become contaminated in the food processing environment. They also demonstrate the need for more studies to investigate the potential sources and scenarios of contamination.

Environmental contamination of *L. monocytogenes* can be very challenging to control because *Listeria* can persist for long periods in seemingly inhospitable environments due in part to its ability to survive a wide range of environmental stresses. For example, it can survive and replicate at temperatures as low as -0.4 $^{\circ}$C through decreased metabolic rate, the expression of cold shock proteins, uptake in cryoprotectants, and changes in cell membrane composition (Bayles & Wilkinson, 2000; Bucur et al., 2018; Ferreira et al., 2014; Gahan & Hill, 2014; International Commission on Microbiological Specifications for Foods, 1996; Ryser & Marth, 2007; Schmid et al., 2009; Suutari & Laakso, 2008). These traits make it a microorganism of interest in foods that are refrigerated for extended durations (FDA, 2020d). Besides cold tolerance, this pathogen can survive adhered to FCS and NFCS through the formation of biofilms that confer increased protection and survival against antimicrobials and other stresses (Doijad et

al., 2015). The presence of other microorganisms like *Pseudomonas putida* and *Lactobacillus plantarum* in these biofilms has been shown to increase the resistance of several strains of *L. monocytogenes* to sanitizers like benzalkonium chloride (BAC) and peroxyacetic acid (PAA) (Saá Ibusquiza et al., 2012; van der Veen & Abee, 2011). Hence, it is critical for food business operators to find and eliminate this potentially persistent pathogen to guarantee the microbiological safety of RTE foods. This relies on the adoption of several measures aimed at preventing food contamination.

Under the Food Safety Modernization Act (FSMA) (*CFR - Code of Federal Regulations Title 21*) environmental monitoring for *L. monocytogenes* is required when the food product is processed without a kill step (e.g. cooking), if the product is exposed to the environment post lethality or before packaging when the product is a collection of RTE products combined to produce RTE food that does not include a kill step, and finally, if the food product is refrigerated and conducive for the growth of *Listeria monocytogenes* (e.g., deli meat, raw cheese/milk, seafood, and sprouts). Routine environmental monitoring involves the microbiological sampling of equipment, tools, personnel, and facilities to detect, eliminate and prevent the growth of niches and to verify the adequacy of control measures (Zoellner et al., 2018). It focuses on the detection of *Listeria* spp. rather than *L. monocytogenes* because *Listeria spp.* is an index for *L. monocytogenes.* This leads to a more robust verification of environmental conditions and a more rapid identification of niches and harborage sites (3M Food Safety, 2019; Grocery Manufacturers Association, 2018). Together with the U.S. zero-tolerance policy for *L. monocytogenes* in food products, this regulatory framework has led to an increasing number of food business operators adopting environmental monitoring as a sanitation verification activity. (3M Food Safety, 2019; Buchanan et al., 2017; FDA, 2020c).

Compliance with Food Safety Modernization Act-Preventive Controls for Human Food Rule (FSMA-PCHF) requires food business operators to make upgrades and even refurbish their manufacturing supply chains from scratch to meet the new standards for anticipating and preventing contamination and recall issues. This can be achieved by large food companies that have the resources to establish an effective verification sampling plan as well as highly trained individuals. For small (companies with fewer than 500 employees) and very small size food establishments (which have less than $1,000,000 in total annual sales of human food) (FDA 2020b), meeting these requirements presents more obstacles due to limited resources and lack of in-house expertise. Moreover, the deadline for small businesses to comply with FSMA-PCHF regulations started in 2017 to continue through 2020. This has placed company owners under substantial pressure to understand and implement these regulations that might be complicated as concerns persist with regards to feasibility especially with the limited resources these companies have to work with (Muhterem-Uyar et al., 2015; Trinetta et al., 2018; Winkler & Freund, 2011).

The objective of this project was to contribute to small and very small food facilities' compliance with the FSMA-PCHF rule. Specifically, we determined the prevalence of environmental *Listeria* spp. and *L. monocytogenes* in food manufacturers in Nebraska and map the distribution of *Listeria* spp. positive sites in food processing operations.

2.2. Materials and methods

2.2.1. Sample collection

From August 2020 to March 2021, environmental samples from three FDA-inspected food processing facilities in Nebraska, i.e., frozen food facility (1 facility), RTE food facility (2 facilities), were analyzed bimonthly for the presence of *Listeria* spp and *L. monocytogenes*. Before the initial sample collection, facilities were visited, mapped and location details were

described to ensure consistency with site sampling upon each visit. Sampling sites were organized into zones following the Draft Guidance for Control of *Listeria monocytogenes* in Ready-To-Eat Foods (FDA, 2017). Sites in Zone 1 represented FCS and Zones 2, 3, and 4 were NFCS in and out of the production area. For sample collection, a kit consisting of cellulose sponge sticks (3M, St Paul, MN), swab-samplers (3M, St Paul, MN), sterile 10 x 10 templates, and a cooler with ice packs, was used to collect 25-30 samples from both FCS and NFCS. Samples were collected 3 to 4 hours into production and shortly before sanitation, representing a time point at which contamination events would most likely be identified. Throughout the study period, each facility was sampled three times. For the selected sites, the pre-hydrated sponge sticks were used to collect samples from FCS and NFCS using a sterile 10x10 cm (100 cm^2) template. Q-tip swab-samplers were used to collect samples from drains with narrow fixed openings. When a template could not be used due to the topography and/or design of the sampling site, e.g., drains, utensils, etc., the surface was swabbed as much as possible to cover its entire area. All sampling devices were applied at least five times in two different directions using both sides of the sponge. The samples were transported back to the University of Nebraska-Lincoln in portable coolers with cool packs within two hours of sample collection and processed immediately upon arrival.

2.2.2. Microbiological and statistical analysis

2.2.2.1. Analysis using 3M™ Petrifilm Environmental *Listeria* Plates

Each sample was tested to detect *Listeria* spp and *L. monocytogenes* according to the manufacturer's instructions (Benesh et al., 2013). For detection using the 3M™ Petrifilm Environmental *Listeria* Plates (PELP; 3M, Saint Paul, MN), 2 ml of D/E neutralizing broth from each sponge stick and swab-sampler was collected with a serological pipette and added to sterile

tubes containing 4 ml of 20% buffered-peptone water (BPW; 3M, Saint Paul, MN) to achieve a 1:2 dilution. The resulting suspension was vortexed, and tubes were allowed at room temperature (20 to30 ˚C) for 60-90 minutes for bacterial cell resuscitation. After resuscitation, the suspension was vortexed and 3 ml of each suspension was transferred to duplicate 3M™ PELP and incubated for 28 hours at 35 ˚C. Red-violet colonies were considered presumptive *Listeria* spp and were sub-streaked on modified oxford agar (MOX; Remel, Lenexa, KS), incubated at 35 ˚C for 24 to 48 hours to observe for typical *Listeria* spp colonies on MOX.

2.2.2.2. Analysis using 3M™ Molecular Detection Assay *Listeria*.

Real-time PCR was performed using the 3M™ Molecular Detection Assay *Listeria* (3M™ MDA) according to the manufacturer's protocol (3M Food Safety, 2020). Briefly, sponge stick and swab samples were enriched by adding Demi-Fraser broth (DFB; 3M, Saint Paul, MN) into sample bags *i.e.,* 100ml of DFB for sponge stick samples and 10 ml of DFB for bags with Q-tip swab-samplers. The samples were homogenized in a stomacher (Seward Stomacher 400C) at 230 rpm for 60 s and incubated at 35 ˚C for 24 hours. After enrichment, a real-time PCR molecular detection of *Listeria* spp gene markers was performed using the 3M™ MDA and the MDA-2 kits for *Listeria* spp. and *L. monocytogenes,* respectively (3M, Saint Paul, MN). Briefly, 20µl of enriched samples were added to lysis tubes and heated at 100 ± 1 ˚C for 15 minutes until a color change from pink to yellow was observed. The lysate was cooled in cooling blocks at 20 to 30 ˚C for 5 minutes. A 20µl volume of lysate was transferred to corresponding reagent tubes to hydrate pellets containing all the PCR reagents. These tubes were transferred into a speed loader tray and labeled into the molecular detection software (3M Molecular Detection System 2.5.0.0) following the sequence in the speed loader tray and the tray was subsequently loaded in the 3M™ MDA device. Software instructions were followed, and readings were obtained after 75

minutes. A standard reference strain, *L. monocytogenes* ATCC 19115 was analyzed simultaneously with environmental samples as a positive control. Kit controls (negative and reagent) were also included during sample analysis as well.

2.2.2.3. Statistical analysis

Data was transferred to GraphPad Prism version 8.0.1 and a Chi-square test performed to determine if there was any association between facility type and frequency of positive samples. Additionally, Fisher's exact test was performed to compare overall prevalence on FCS versus NFCS.

2.3. Results and discussion

2.3.1. Prevalence of *Listeria monocytogenes*/*Listeria* spp

The combined prevalence of *Listeria* spp in all three facilities was 5.3% (14 of 266). This overall prevalence falls within the range of prevalence of *Listeria* spp (1.6%- 36%) reported in FDA-regulated facilities (Reinhard et al., 2018). Other studies have reported an overall prevalence that was either similar or higher than the observed prevalence in our study, but they all fall within the range for FDA-regulated facilities (Simmons et al., 2014; Viswanath et al., 2013; Williams et al., 2011). Variation in prevalence across different facilities has been attributed to plant-specific sanitation procedures and food safety policies hence, it is critical to developing plant-specific *Listeria* control strategies (Lappi et al., 2004).

Listeria monocytogenes was not detected in any of the facilities during our study. Similar observations were made by Williams et al., (2011) where *Listeria* spp was more prevalent than *L. monocytogenes* in small and very small RTE meat processing plants. Other studies (Estrada et al., 2020; Kovačević et al., 2009; Viswanath et al., 2013; Simmons et al., 2014) have observed a higher prevalence of *L. monocytogenes* than *Listeria* spp. For example, Simmons et al., (2014)

observed a prevalence of 5.3% (237 of 4503) for *Listeria* spp compared to 9.5% (428 of 4503) in retail deli environments. While detection of *Listeria* spp is often used as a good indicator of potential *L. monocytogenes* contamination, these studies show that testing for *L. monocytogenes* in certain environments could be an appropriate strategy to control *L. monocytogenes* than testing for *Listeria* spp as an index (Estrada et al., 2020; Kovačević et al., 2009; Viswanath et al., 2013; Tompkin et al., 1999). Since *L. monocytogenes* was not detected in any of the facilities in our study facilities, it can be suggested that *Listeria* spp was a reliable indicator of potential *L. monocytogene*s contamination.

Most of our samples were collected during operations which may have contributed to the observed overall prevalence of *Listeria* spp. A study by Reinhard et al., (2018) observed a lower prevalence of *Listeria* spp during preoperational sampling than during operations. This indicates that the timing of sample collection influenced the observed prevalence in our study. Thus, it underscores the importance of timing during sample collection. Additionally, other *Listeria* spp like *L. innocua* have may outgrow *L. monocytogenes* during enrichment, hence masking its presence (Beumer et al., 1996; Oravcová et al., 2008). This could also be a reason for the higher prevalence of *Listeria* spp was than *L. monocytogenes*. Overall, it is important to consider all these methodological issues when designing a *Listeria* sampling program.

2.3.2. Prevalence of *Listeria* spp on FCS and NFCS

Of the 266 total samples collected, 2.7% (2 of 73) were from FCS and 6.2% (12 of 193) from NFCS (Table 2.1). There was no significant difference between the prevalence of *Listeria* spp positive samples for FCS and NFCS (P = 0.36; Fisher's exact test). This was not consistent with previous data that have demonstrated a significantly higher prevalence of *Listeria* spp on NFCS than on FCS (Tompkin, 2002; Lappi et al 2004). However, NFCS had a higher frequency

of *Listeria* spp positive samples than FCS and this has been reported in prior studies. For instance, Hoelzer et al., (2011) reported a higher frequency of occurrence of *L. monocytogenes* positive samples on NFCS in retail, dairy, raw meat, seafood, and produce handling establishments (17%; 293 of 1731) but FCS had a lower frequency of positive *Listeria monocytogenes (*3.6%; 45 of 1250) samples. In addition, a significant difference between the prevalence of *Listeria monocytogenes* on FCS food contact and NFCS nonfood-contact surfaces could be observed. Such differences in frequency or prevalence of *Listeria* spp are due to increased exposure of FCS to sanitizers than NFCS. Moreover, the absence of sanitation verification programs such as environmental monitoring has also been reported to account for the higher prevalence of *Listeria* spp as facilities cannot assess the efficacy of sanitation activities for the control of *Listeria* spp (Jorgensen et al., 2020; Ruiz-Llacsahuanga et al., 2021).

2.3.3. Distribution of *Listeria* spp in facilities

Overall, the prevalence of *Listeria* spp varied across facilities. It ranged from 4.3% - 5.9% with B having the highest prevalence of all 3 facilities (5.9%; 5 of 85) (Figure 2.1). There was no association between facilities and the number of positive samples (P = 0.89). However, examining facility characteristics provided information that may support variations observed in *Listeria* spp prevalence among facilities.

2.3.3.1. Plant A

Plant A (Figure 2.2) manufactured a variety of frozen pies (ready-to-bake) for retail and (supermarkets and convenience stores). Production employees were responsible for cleaning production tables, equipment, and floors before the start of operations and after every break throughout the day. After production ends, equipment, utensils, and floors are cleaned. Employees working in the main food processing zone were required to use aprons, appropriate

personal protective equipment (PPE) i.e., hair and beard nets, and dedicated footwear. Personnel was also instructed on proper handwashing procedures and glove use. Facility A had controlled traffic of personnel and equipment including other barrier measures like the use of PPE that was readily available in a dedicated transition area. PPE was to be worn before entering the main processing area where pies were manufactured. Overall, facility A had more targeted control strategies and had a sanitary design of equipment and the facility. Controlled traffic flow, the use of PPE, and sanitary design have been shown to contribute to reduced cross-contamination and prevalence of *Listeria* in food establishments (Lappi et al., 2004). This may have been the case with facility A. Examples of sites in this plant that were positive for *Listeria* spp were the lower shelf of an assembly table (n=1) (zone 2), cleaning equipment *e.g.*, squeegee (n=1) (zone 3), warehouse floor (n=1) (zone 4), and forklift tires (n=1) (zone 4). All these sites were NFCS that were either in direct contact with the floor or close to the floor (lower shelf of the table) (Table 2.1). Prior studies have shown floors to be associated with the high prevalence of *Listeria* spp suggesting that more attention needs to be dedicated to cleaning and sanitizing these sites (Hoffman et al., 2003; Lappi et al., 2004). The detection of *Listeria* spp on forklift tires suggests the potential of cross-contamination from the external environment. Tires or wheels on mobile NFCS can serve as points for cross-contamination (Estrada et al., 2020; Simmons et al., 2014; Williams et al., 2011). Wheel baths and cross-contamination training of personnel are targeted control strategies that have been shown to control *Listeria* spp in these sites (Lappi et al., 2004). As concerns sites in zone 2 like the lower shelf of the stainless-steel table, viable aerosols generated during cleaning could have accounted for the migration of *Listeria* spp from zone 3 or 4. Previous studies have made similar observations hence underscoring the need for enhanced

cleaning and sanitation activities in zones 3 and 4 (Lekroengsin et al., 2007; Ruiz-Llacsahuanga et al., 2021; Saini et al., 2012).

2.3.3.2. Plant B

Plant B (Figure 2.3) manufactured RTE sandwiches, salads and, snacks, for vending machines companies. This facility had older infrastructure including some sanitary design issues like an unleveled floor plan that did not allow for water to flow to drains as such, pooled water could be seen in some parts of the main processing area. In this plant, employees were responsible for cleaning after each production cycle and after production. This was the only facility that had a *Listeria*-positive sample from FCS (zone 1) i.e., meat slicer blade (n=1) and the gloves of the meat slicer operator (n=1). Other locations in facility B that were positive for *Listeria* spp included drains (n=2) and, the floor in the walk-in cooler (n=1) all in zone 3 (Table 2.1). *Listeria* spp was not detected in zones 2 and 4 in this facility. All positive samples from this facility came from the prep kitchen area where raw materials are prepared and sent to the assembly area for assembly and packaging. The assembly area was in a separate part of the facility building. Some raw materials commonly handled in the prep area included raw produce, processed cheese, and RTE processed meats. Plant B was the only facility that had produce as a raw material. Fresh produce is normally received directly after harvest and processed rapidly to facilitate refrigeration with minimal antimicrobial interventions (John et al., 2020). Additionally, prior studies have shown produce environments to have a *Listeria* spp prevalence of up to 12% (126 of 1,092) in soil and 90% (47 of 52) in water (Weller et al., 2015). Because fresh produce is handled with little to no antimicrobial interventions and comes from environments that have been shown to have a high prevalence of *Listeria* spp, it can be suggested that fresh produce contributed to the contamination and the high prevalence of *Listeria* spp in this facility compared

to the others. This finding is consistent with other studies where raw materials like raw produce were shown to contribute to the contamination of FCS (Lappi et al., 2004). There was insufficient control of the traffic of mobile NFCS and personnel in the main processing areas. In addition, there were insufficient hygienic barriers to prevent the introduction and spread of *Listeria* spp in high-risk areas. For instance, in the prep kitchen, personnel operating the deli meat and cheese slicer could be observed moving from slicer tables to the produce prep area where raw produce was handled. The uncontrolled movement and the absence of hygienic barriers could have contributed to the contamination of FCS like employee gloves and the deli meat slicer. Previous studies have demonstrated that uncontrolled traffic and insufficient hygienic barriers lead to a higher prevalence of *Listeria* spp in processing through cross-contamination. For example, (Lappi et al., 2004) reported that uncontrolled movement of personnel and equipment and insufficient hygienic barriers contributed to the introduction and spread of *Listeria* spp in food RTE smoked salmon establishments. In this study, a significant decrease in the prevalence of *Listeria* spp in finished product areas and NFCS was observed after the implementation of targeted control strategies such as improved control of traffic, and installation of door foamers as hygienic barriers. Another study in fresh produce handling facilities reported similar findings (Estrada et al., 2020). Besides traffic, cross-contamination due to the proximity of contaminated sites to FCS was also observed to be an issue. As an example, the proximity of a *Listeria*-positive drain to the meat slicer table could have contributed to the contamination of the meat slicer through viable aerosols generated and spread during cleaning operations especially with a spray hose. Hence, optimization is required during cleaning operations to limit the generation of viable aerosols (Saini et al., 2012). *Listeria* spp was detected in other sites like the drain of the mop storage area (n=1) and the floor of one of the walk-in

coolers (n=1). Drain sites and cold storage have been associated with a high prevalence of *L. monocytogenes* and *Listeria* spp (Estrada et al., 2020; Hoffman et al., 2003; Lekroengsin et al., 2007). These sites could readily serve as points of cross-contamination onto final products (Rørvik et al., 1997). Overall, the frequency of occurrence of *Listeria* spp positive samples was barely higher on NFCS (n=3) than on FCS (n=2). Possible control strategies for *Listeria* spp in this facility could include employee training on cross-contamination and sanitation, change in slicer sanitation procedure, traffic control, and door foamers with sanitizer installed at the entrance of the processing areas (Lappi et al., 2004). Remodeling of infrastructure with considerations on sanitary design is another strategy that can contribute to lowering the prevalence of *Listeria* spp in this facility (Lappi et al., 2004).

2.3.3.3. Plant C

Plant C (Figure 2.4) manufactured RTE sandwiches for convenience stores and vending machines across the U.S. This plant had older infrastructure that did not allow for sanitary design. Personnel was responsible for cleaning after every shift and at the end of the day. They were also required to use dedicated aprons, hairnets, and gloves upon entry into the main production area. The traffic of personnel was controlled in this facility, but this did not include mobile NFCS. All *Listeria*-positive samples in this facility originated from zone 3. These included drains (n = 1), antifatigue mat (n = 1), leg of three-compartment sink (n =1), wheels of metal platform trolley (n = 1) and the floor (n = 1) (Table 2.1). The detection of *Listeria* spp on mobile NFCS like the trolley was indicative of a potential for cross-contamination from the external environment as this platform was used to carry heavy items from outside into the main processing area (Estrada et al., 2020; Lappi et al., 2004). We did not observe a very high frequency of occurrence of *Listeria* spp positive samples from the drain and floor sites but our

data suggest the association of *Listeria* with these sites (Estrada et al., 2020; Hoffman et al., 2003; Lekroengsin et al., 2007; Saini et al., 2012). Findings from our study reaffirm the importance of preventing *Listeria* spp colonization of facilities by scheduling regular and adequate cleaning of floors and drains as they are a primary site of contamination. It also highlights the importance of targeted interventions like the controlled flow of equipment and the use of hygienic barriers as ways to prevent the colonization of the food facility and possible cross-contamination to FCS.

2.3.4. 3M™ Molecular Detection Assay *Listeria*/3M™ Petrifilm Environmental *Listeria* Plates

The frequency of *Listeria* spp positive sites was higher when samples were analyzed with 3M™ PELP (4.9%; 13 of 266) than with the 3M™ MDA(0.3%; 1 of 266) (Table 2.1). In addition, the positive samples on the 3M™ PELP were negative on 3M™ MDA and vice versa. This was not consistent with prior validation studies of the 3M™ MDA and 3M™ PELP. For example, Abatcha et al., (2020) analyzed 178 samples obtained from fresh leafy vegetables, chicken, and their related environments with both the 3M™ MDA and the 3M™ PELP, and the results were compared with the EN ISO 11290-1 reference method. Overall, the 3M™ MDA *Listeria* showed high specificity (99.3%), accuracy (97.2%), and nearly complete agreement (k = 0.911) with the standard EN ISO 11290-1 method. The 3M™ PELP showed higher specificity (100%), an accuracy of 96.1%, but a slightly lower agreement (k = 0.894) with the standard EN ISO 11290-1 method compared to the 3M™ MDA. Overall, in this study, the 3M™ MDA detected more true positive samples (42 of 178) than the 3M™ PELP (40 of 178) and almost all samples that were positive on the 3M™ MDA were positive on 3M™ PELP. The conclusion was that both methods provided fast and reliable results for monitoring and detection of *Listeria* in the food processing plant environment. Studies carried out by (Horter & Lubrant, 2004;

Vongkamjan et al., 2015) reported similar observations and arrived at the same conclusions. However, (Abatcha et al., 2020) obtained false positive (1 of 178) and false-negative samples (5 of 178) on 3M^{TM} MDA and suggested that such outcomes affect the sensitivity and specificity of this method. Current studies have suggested that false positives can occur because of a higher number of primers ranging between 4 and 6 compared to conventional PCR. This higher concentration may contribute to the development of a non-specific amplification induced by the formation of dimers that can result in a false positive outcome (Wang et al., 2015). Another suggested reason is the single enrichment step that lasts for up to 30 hours and does not allow for resuscitation of stressed *Listeria* to levels that facilitate detection (Vongkamjan et al., 2015). A study by (Fortes et al., 2013) observed that the low levels of *Listeria* spp after a single enrichment step contribute to decreased sensitivity of the 3M^{TM} MDA and can result in a false negative outcome. Additionally, insufficient quantities of lysate transferred to tubes have been reported to account for false negatives in the 3M^{TM} MDA (Loff et al., 2014). Finally, the amplification of genetic material from dead cells can also account for false positives on the 3M^{TM} MDA.

Because we observed a single positive sample on the 3M^{TM} MDA that was not positive on the 3M^{TM} PELP, it can be suggested that our sample was a false positive possibly due to amplification of dead *Listeria* spp genetic material or because of non-specific amplification due to the formation of dimers. This could explain why the positive sample on the 3M^{TM} MDA was not positive on 3M^{TM} PELP. It is also possible that the single-step enrichment did not allow for the resuscitation of sub-lethally injured cells. Regardless, additional validation studies are needed to confidently draw conclusions on the performance of the 3M^{TM} MDA and the 3M^{TM} PELP especially when it comes to samples from RTE environments. Current validation studies for

these two methods use environmental samples from plants that will most likely have high levels of *Listeria* spp/*Listeria monocytogenes* such as produce, poultry, or seafood environments (Abatcha et al., 2020; FORTES et al., 2013; Vongkamjan et al., 2015) so it is important to carry out this study in RTE food environments to cover this knowledge gap.

2.4. Conclusion

Production environments, operations, cleaning, and sanitation practices varied across all three participating facilities. *Listeria* spp was detected in the environment of all these facilities. Conversely, *L. monocytogenes* was not detected in any of the facilities. Though no significant difference in prevalence was observed, there was still some variation in prevalence with B being the facility with the highest among all three plants. The sanitary design of the facility, the potential for cross-contamination associated with uncontrolled traffic of mobile NFCS, and lack of hygienic barriers represented major challenges for controlling *Listeria*-contamination. Just like other environmental monitoring studies, NFCS had a higher frequency of *Listeria*-positive samples than FCS hence, highlighting the need for *Listeria* control strategies in these areas. Facilities were provided with reports of sample analyses after each visit and these included recommendations on strategies to control *Listeria* spp. This data was also going to support facilities' compliance with the FSMA-PCHF rule. Overall, our study demonstrates that environmental monitoring can be used as a powerful tool to detect *Listeria*-contaminated sites, points of entry and identify situations that lead to cross-contamination thus informing *Listeria*-control strategies.

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Tables

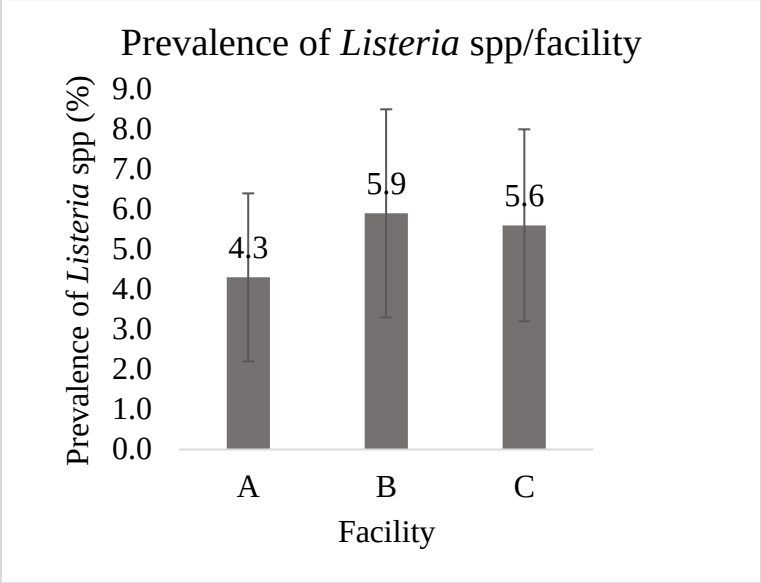
Table 2. 1. Frequency of *Listeria* spp positive samples for each detection method

	Facility	A	B	C	Total
	Product	Frozen food	RTE food	RTE food	
	No. (%) of positives on PELP	3(3.2)	5(5.9)	5(5.6)	13 (4.9)
	No. (%) of positives on MDS	1(0.3)	0	0	1 (0.3)
	No. of samples/ facility	92	85	89	266
***Listeria* spp positive samples per zone**	**Zone 1**	0	2	0	2 (2.7)[a]
	Zone 2	1	0	0	12 (6.2)[b]
	Zone 3	1	3	5	
	Zone 4	2	0	0	
	Sites	Table, squeegee, floor, forklift	Glove, meat slicer, drains, floor	Sink, mat, drain, floor, trolley	

a and b (superscripts) represent the cumulative prevalence of *Listeria* spp on FCS and NFCS, respectively

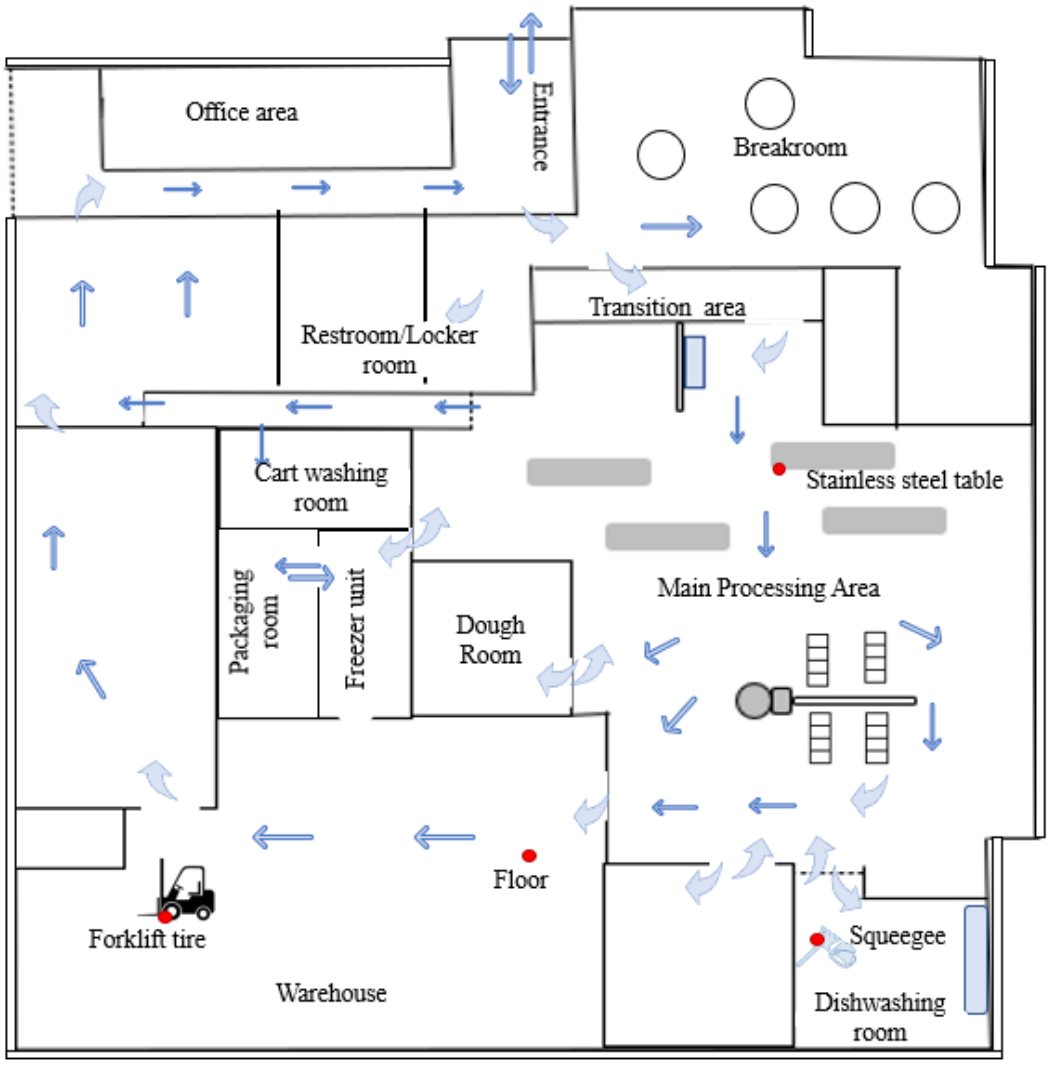
Figures

Figure 2. 1. Prevalence of *Liste*ria spp across facilities.



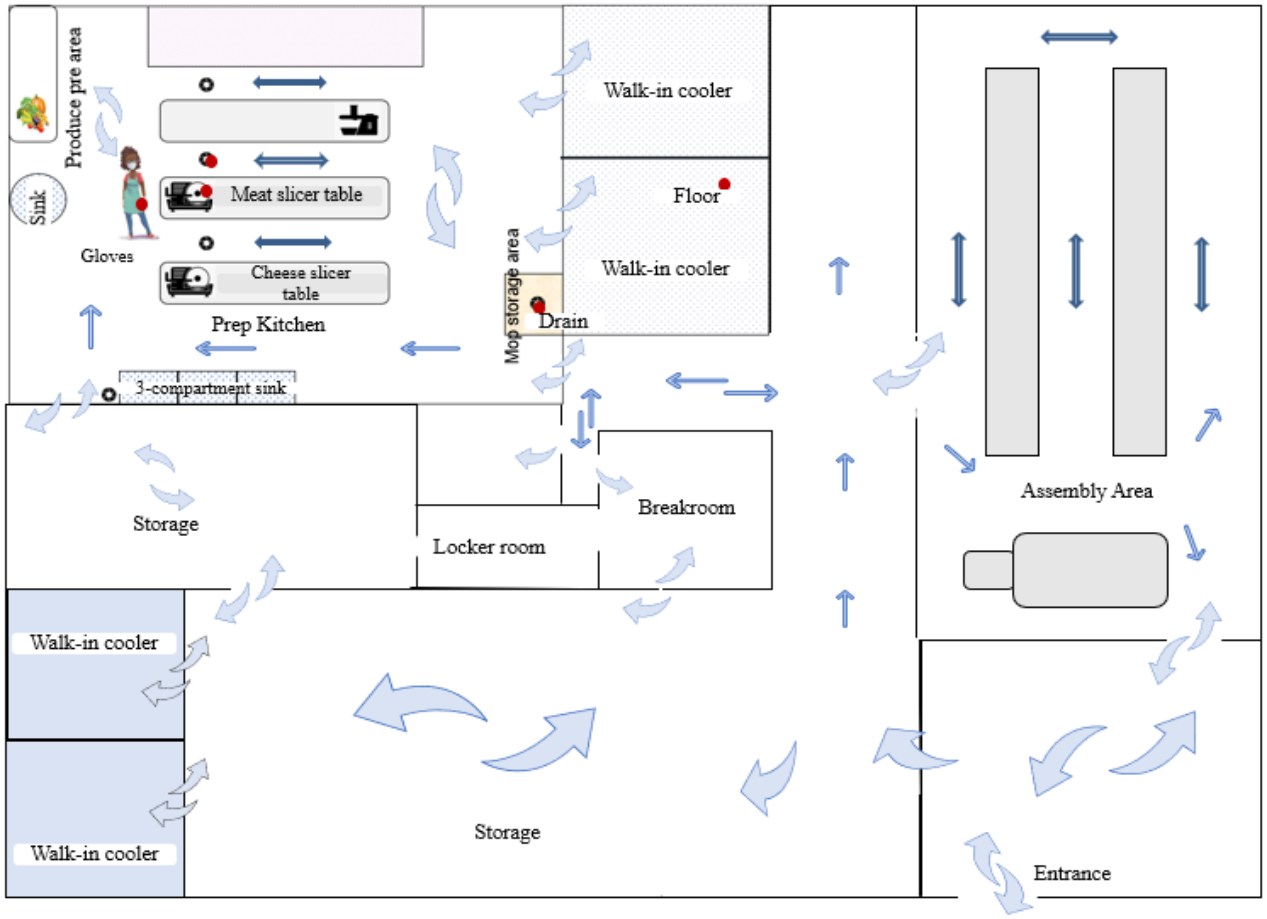
Bars represent standard error of proportions.

Figure 2. 2. Plan of Facility A



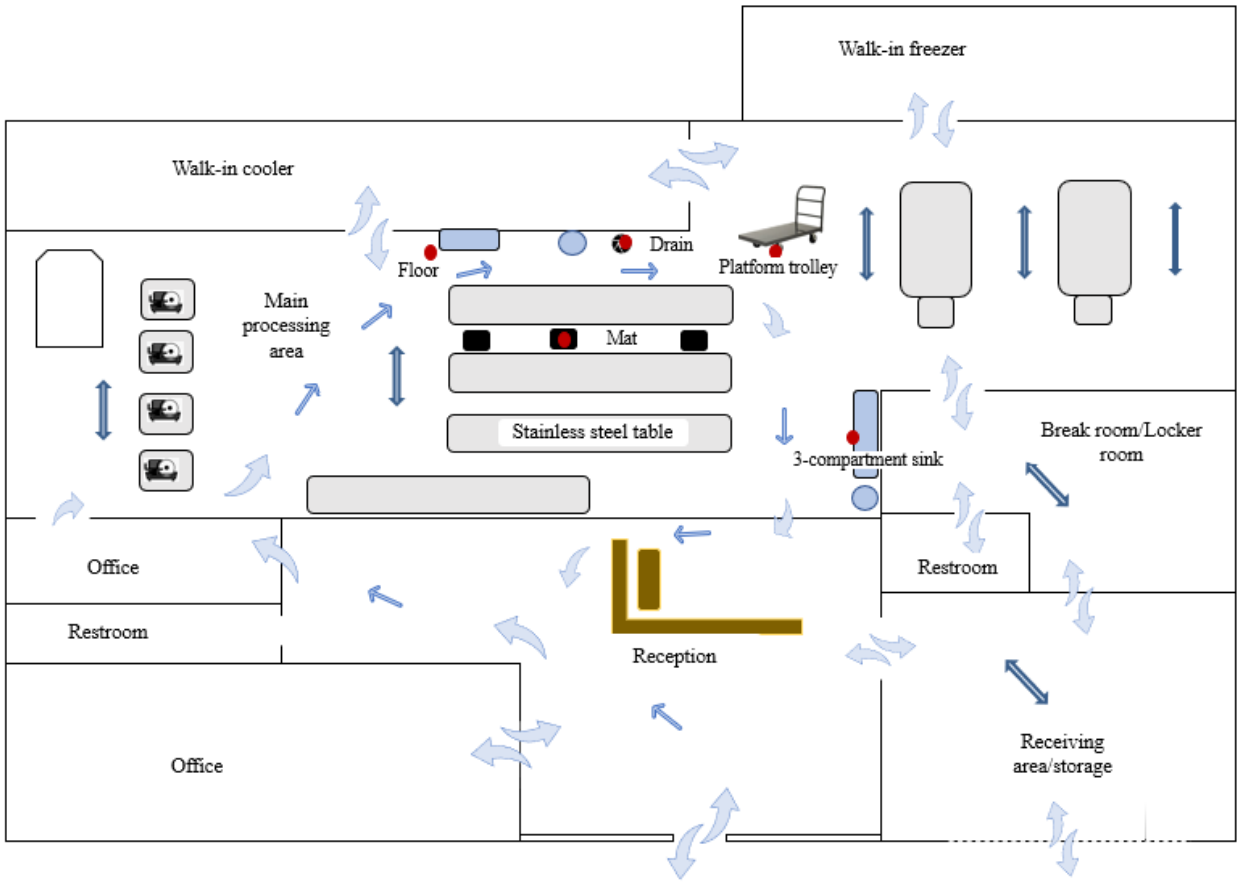
Arrows indicate movement within the facility. Red dots are sites that were positive for *Listeria* spp.

Figure 2. 3. Plan of Facility B



Arrows indicate movement within the facility. Red dots are sites that were positive for *Listeria* spp.

Figure 2. 4. Plan of Facility C



Arrows indicate movement within the facility. Red dots are sites that were positive for *Listeria* spp.

CHAPTER 3. EFFICACY OF OZONATED WATER FOR THE DECONTAMINATION OF FOOD CONTACT SURFACES SOILED WITH ORGANIC MATTER

Abstract

A good sanitation program prevents contamination from environmental pathogens and contributes to facilities' compliance with regulations. Adequate sanitation can be achieved through sanitation technologies like ozone technology. Ozone is a viable alternative to other sanitizers like chlorine because of its high oxidizing properties and its effectiveness against bacteria. Despite the advantages of ozone, there still exist limitations to its efficacy notably due to factors that affect microbial sensitivities like organic matter, target microorganisms, the physical state of ozone, etc. Because its efficacy depends on several factors, it may impact the selection of a sufficiently effective dose. As a result, there is a need for more comprehensive information regarding its efficacy under different conditions. This study aimed to evaluate the efficacy of ozonated water for the decontamination of *Listeria* on food contact surfaces. For this study, stainless steel and polypropylene coupons constructed to 10 x 10 cm were conditioned with a meat emulsion made from uncured deli turkey breast and inoculated with *Listeria monocytogenes* strain ATCC 19115. Other experiments were performed with *L. innocua* strain ATCC 33090 separately. In addition, clean coupons were also included for experiments with *L. innocua*. Inoculated surfaces were exposed to 10 ppm of ozonated water for 15, 30, and 45 seconds, respectively. Tap water was included as a treatment. There were no significant differences in reductions attributed to ozonated water compared to tap water washing. However, reductions of *L. innocua* on soiled stainless steel was significantly higher than on clean surfaces($P = 0.01$). Similarly, *L. innocua* reductions were numerally higher on soiled polypropylene though not significantly different from clean coupons. Spraying applications may

have influenced bacterial reduction from surfaces by dislodging rather than actual inactivation. In addition, the soil system with deli turkey may have not provided sufficient soil (grease) to reduce the efficacy of ozonated water resulting in similar reductions on both soiled and clean surfaces. Overall, data suggest that cleaning may be effective at reducing transiently attached *Listeria* form FCS.

3.1. Introduction

The ability of *L. monocytogenes* to survive and grow at refrigeration temperatures and its ubiquity has resulted in food business operators dedicating extensive resources and implementing measures to prevent and control its presence in food. Some of these measures are geared towards maintaining the food processing environment in a sanitary condition through good manufacturing practices (GMP) or sanitation standard operating procedures (SSOP). Some establishments also include additional programs for the verification of control of this pathogen. These programs will typically involve the microbiological sampling of food contact surfaces (FCS) and nonfood-contact surfaces (NFCS) (Reinhard et al., 2020; Tompkin, 2002). Food processing establishments will generally investigate the root cause of *Listeria* contamination in the environment and then initiate an intensive cleaning and frequent sampling of the affected area including processing equipment or the immediate environment (Reinhard et al., 2020). Cleaning and sanitation typically consist of using the appropriate detergent combined with mechanical scrubbing to remove soil or debris. The goal is to minimize organic matter so that disinfection can be effective (U.S. Department of Agriculture-Animal and Plant Health Inspection Service, 2018). Chemical sanitizers are subsequently applied to destroy or eliminate species of microorganisms that are of concern.

Ozone has to potential for use as a sanitizer throughout the food production supply chain to control bacteria of human health concern. It is increasingly being used in industry for its antimicrobial properties and its recognition as generally recognized as safe (GRAS) chemical (Sopher et al., 2009). The high oxidation potential of ozone makes it an attractive alternative to traditional sanitizing agents like chlorine. Because ozone rapidly decomposes, it is not persistent and does not leave any toxic residues making it a suitable alternative for users concerned about the environment. It has also been shown to be effective for pesticide residue reduction (Ong et al., 1996), food preservation, shelf-life extension, and equipment sterilization (Hampson, 2000).

Ozone inactivates bacteria through a complex process that damages various cell membrane constituents like lipopolysaccharides and lipoproteins. It also destroys cell content constituents such as enzymes and nucleic acids. Besides molecular ozone, free radicals such as hydroperoxyl, hydroxyl, and superoxide, which are produced as it decomposes also play a role in bacterial inactivation. The bacterial cell is killed due to disruption of the cell membrane leading to cell content leakage. Cell lysis is an effective inactivation mechanism compared to others sanitizing agents that need to get into the cell membrane to be effective. Because cell death with ozone is through cell lysis, ozone use cannot lead to the resistance of microorganisms (Pascual et al., 2007). Prior studies have demonstrated the use of ozonated water to effectively decontaminate different kinds of FCS. For example, Greene et al., (1993) used ozonated water to decontaminate stainless steel surfaces inoculated with UHT milk that had been contaminated with *Pseudomonas florescens* and *Aeromonas faecalis.* Surfaces were treated with 0.5 ppm of ozonated water and held for up to 10 minutes resulting in a 5.6 log reduction of *P. florescens* and a 4.4 log reduction of *A. faecalis.* In another study by Megahed et al., (2018), the use of aqueous ozone resulted in a reduction below limits of detection (ca. 6.4 $\log_{10}$ reduction) when it was

applied as wash water at a concentration of 4 ppm and above onto plastic surfaces inoculated with manure-based pathogens. A similar outcome was observed on stainless steel surfaces. This study reported a lower reduction of manure-based pathogens on wood surfaces (ca. 2.0 $\log_{10}$ reduction) upon exposure to 4ppm of aqueous ozone for up to 8 minutes. This was attributed to the fact that wood-based materials are complex with high molecular weight components and release ozone reactive substances (volatile organic compounds) that consume ozone before it reaches microbes in the irregular pores of the wood. All materials used in this study displayed resistance to damage from aqueous ozone.

Most FCS employed in industry are plastic materials like polypropylene or stainless steel (304 and 316) (Skåra & Rosnes, 2016). These materials perform well in the presence of ozone and their resistance to corrosion from oxidation is good or excellent. This makes ozone a suitable sanitizer for decontaminating these surfaces (Leusink, 2018; Pascual et al., 2007).

Despite the advantages of ozone, there still exist limitations to its efficacy. First, microorganisms possess different sensitivity to ozone which depends on factors like product type, target microorganisms, the initial level of contamination, physiological state of bacteria, the physical state of ozone, and the type of organic material (Miller et al., 2013; Restaino et al., 1995). Experimental conditions also account for varying antimicrobial efficacy of ozone (Pirani, 2010). Because its efficacy depends on several factors, it may cause limitations in the selection of a sufficiently effective dose (Brodowska et al., 2018). As a result, there is a need for more comprehensive information regarding its efficacy under different conditions. Hence, this study aimed to evaluate the efficacy of ozonated water for the decontamination of *Listeria* on food contact surfaces

3.2. Materials and methods

3.2.1. *Listeria* strains and inoculum preparation

The bacterial strains: *Listeria monocytogenes* strain ATCC 19115 and *Listeria innocua* strain ATCC 33090, used in this study were obtained from the American Type Culture Collection (ATCC). These strains were selected because they are commonly available as quality control strains (Reinhard et al., 2020). Inoculum preparation was adapted from (Reinhard et al., 2020) with some modifications. Working inoculum suspensions were prepared from frozen culture (-80 °C). Briefly, frozen culture was streaked onto brain heart infusion agar plate (BHIA; Remel, Lenexa, KS) and incubated at 35 °C for 24 hours. A single isolated colony was transferred to a 250 ml Erlenmeyer flask containing 50 ml of BHI broth (BHIB; Oxoid, Basingstoke, UK). The flask was incubated on a shaking platform incubator (Thermo Scientific, MaxQ600, Manetta, OH) at 250 rpm for 24 hours at 35 °C. Cells were harvested by centrifugation at 1068 x g for 20 minutes (ThermoFisher Scientific; Sorvall Legend X1R, Osterode arm Harz, Germany) at room temperature (22 °C, RT) (Limoges et al., 2020; Nicholas et al., 2013; Reinhard et al., 2020). The pellets were re-suspended in 10ml of BHIB, and the resulting inoculum suspension was serially diluted in 0.1% buffered peptone water (BPW; Sigma-Aldrich, St.Louis, MO) and plated on modified oxford agar (MOX; Remel, Lenexa, KS) to verify cell concentration (Nicholas et al., 2013; Reinhard et al., 2020). The target level was 9 $\log_{10}$ CFU/ml. Experiments were performed with the two bacterial strains separately.

3.2.2. Test Coupons.

Two different materials used in food processing facilities were used in this study. These were stainless steel 304 (River Metals Products, Lincoln, NE) and polypropylene (Electron Microscopy Sciences, Hatfield, PA) all constructed to 10x10 cm coupons (Reinhard et al., 2020).

Before inoculation, coupon surfaces were treated with 70% ethanol, rinsed with deionized water, and allowed to dry out. Each coupon was wrapped separately in aluminum foil and autoclaved at 121°C for 15 min to sterilize surfaces (de Candia et al., 2015; Reinhard et al., 2020).

3.2.3. Coupon conditioning and inoculation

Uncured, deli turkey breast was used as organic matter for coupon surface conditioning. The deli turkey breast was manufactured in the University of Nebraska-Lincoln Loeffel Meat Laboratory (Lincoln, NE,). The formulation contained 80% boneless skinless turkey breast, 17.3% water, 1.6% salt, 0.8% sugar, and 0.3% sodium phosphate. The measured fat and protein content was 0.17% fat and 21.6% protein. Organic matter was prepared from the deli turkey breast as previously described by Gram et al., (2007) and Birk et al., (2004) with some modifications. Briefly, deli turkey breast that had been frozen at -20 ˚C was thawed at 4 ˚C overnight. To prepare the organic matter, a 50 g portion of the deli turkey was cut and placed in a sterile sample bag with a filter (Nasco Whirl-Pak, U.S). Subsequently, 100 ml of 0.1% BPW (Sigma-Aldrich, St.Louis, MO) was added into the bag. This was transferred to a stomacher (Seward Stomacher 400C) and homogenized for 2 minutes at 230 rpm to obtain an emulsion with 1:2 dilution. Before inoculation, the filtered organic matter was spread to cover the entire area of coupons that had been laid flat in a sterile biosafety cabinet at room temperature (Birk et al., 2004; Brown et al., 2014; Hua et al., 2019; Limoges et al., 2020).

Duplicate coupons of each material were inoculated by spot applying the inoculum suspension immediately after surfaces were conditioned with the organic matter. Coupons were each inoculated with 0.1ml of inoculum suspension excluding 2 mm of the edge to a target density of approximately 6 $\log_{10}$ CFU cm^2. Inoculated coupons were allowed to dry out for 1 hour at room temperature under the biosafety cabinet (de Candia et al., 2015; Hua et al., 2019).

Other experiments were also performed separately with clean coupons (without organic matter). In these experiments, only *L. innocua* was used to inoculate coupon surfaces.

3.2.4. Ozonated water treatment

Inoculated surfaces were treated with ozonated water generated from the Viriditec aqueous ozone system (TetraClean/CleanCore Technologies, Omaha, NE). This system uses proprietary nanobubble technology to combine water and ozone yielding ozonated water. Ozone was bubbled in water up to a concentration of 10 ppm. This concentration was selected based on feedback from industry where 1 ppm was recommended to effectively reduce bacterial load. The recommended level was used for the decontamination of *Salmonella* in raw poultry but did not result in significant reductions (Cano et al 2021). So, the concentration was increased to 10ppm, and the same concentration was used for this study. Ozonated water concentration was measured by a sensor on the equipment and displayed on a digital dissolved-ozone monitor (Q45H, Analytical Technology, Inc., Collegeville, PA). The concentration displayed on the appliance was verified using the CHEMets® kit (CHEMetrics, Midland, VA). For our sanitation treatments, all coupons were spray washed with ozonated water generated *in situ* from the appliance at a flow rate of 3.79 liters min^{-1} at different exposure times; 15, 30, and 45 seconds, respectively. After treatment, the coupons were swabbed with pre-hydrated (neutralizing buffer) polyurethane sponges (World Bioproducts, Libertyville, IL) to collect any surviving cells. Water treatment was also included in this study wherein tap water at room temperature and flow rate of 3.79 liters min^{-1} was used to treat surface at the same exposure times as with aqueous ozone.

3.2.5. Microbiological analyses

Neutralizing broth from the sponge samplers was used to prepare serial dilutions and aliquots plated on modified Oxford (MOX; Remel, Lenexa, KS) agar plates. After incubation at

35 ˚C for 48 hours, typical *Listeria* colonies were counted and recorded to obtain counts for *Listeria* on each coupon (Franklin et al., 2004).

3.2.6. Experimental design and statistical analysis

Data were arranged in a complete randomized design with a factorial arrangement of treatments. Surfaces inoculated with *L. innocua* had three factors that included exposure time, surface condition, and sanitation treatment. Coupons were treated with water and aqueous ozone and sampling was done at three-time points (15, 30, and 45 seconds) for clean and conditioned/soiled surfaces. For surfaces inoculated with *L. monocytogenes*, there were two factors (time and sanitation treatment). Experiments for *L. monocytogenes* were done only with soiled coupon surfaces. All experiments were repeated three times. Log_{10} transformations and reductions were performed on plate counts, and results were reported in CFU cm^{-2}. Data were imported into R Studio version 4.0.2 and analyzed by analysis of variance (ANOVA) with Tukey's pairwise comparison. Significant differences were reported where $p \leq 0.05$. Material and bacterial combinations were analyzed separately.

3.3. Results and Discussion

There was no significant difference in log_{10} reduction of *L. innocua* between water and ozonated water on stainless steel (P = 0.33; Table 3.1). However, the overall reduction of *L. innocua* on soiled surfaces was significantly higher than on cleaned surfaces (P = 0.01; Table 3.1) particularly at longer contact times where reductions were up to 2.61 log_{10}. Relative to initial inoculum (6 log_{10} CFU) reductions on stainless steel ranged between >90 and >99%.

For polypropylene coupons, there was no significant reduction of *L. innocua* between ozonated water and water (P=0.46) for both cleaned and soiled surfaces (Table 3.2). Longer contact times resulted in higher numerical reduction especially on soiled surfaces where a 3.21 log_{10} reduction

(>99.9% reduction relative to initial inoculum) was achieved with water suggesting water can be sufficient to remove transiently attached *Listeria* on soiled food contact surfaces. Reductions on soiled surfaces were numerically higher than on cleaned surfaces though not statistically significant.

For surfaces inoculated with *L. monocytogenes*, there was no significant difference in log reduction between water and ozonated water on both polypropylene and stainless-steel surfaces (P=0.47 and P= 0.86 respectively: Table 3.3). There were also some inconsistencies in data notably with reductions on polypropylene due to technical issues with the ozonation unit that resulted in the instability of ozonated water concentration. Although both materials were analyzed separately, reductions with ozonated water on polypropylene were slightly higher (between 99 and >99.9% reduction) than on stainless steel (between 90 and 99 % reduction) relative to the initial inoculum level. Overall, the data suggest water could be effective at eliminating *L. monocytogenes* transiently attached to soiled food contact surfaces.

A major finding in this study was that water was as effective as was ozonated water at removing transiently attached *Listeria* on both stainless-steel and polypropylene surfaces. This is not consistent with previous data. For example, Greene et al., (1993) showed that concentrations as low as 0.5 ppm of aqueous ozone could reduce 4.4 to 5.6 $\log_{10}$ of bacteria on stainless steel surfaces. Other authors like Gatima et al., (2021) also observed significant reductions (1.1 $\log_{10}$) of *L. innocua* when 2 ppm of ozonated water was used to decontaminated stainless-steel surfaces for a 60-second contact time. Contrary to some previous reports, shorter contact times were used in our study. For example, Megahed et al., (2018) used a contact time of 2 minutes at 9 ppm of ozonated water resulting in a total kill (ca. 7.1 $\log_{10}$) of manure-based pathogens (e.g., *L. monocytogenes, Salmonella* spp, and *E. coli*) on stainless steel and a level of 4 ppm of ozonated

water for 2 minutes to result in total kill (ca. 6.4 $\log_{10}$) of manure-based pathogens on polypropylene surfaces. Another study by Gatima et al., (2021) showed significant reductions of *L. innocua* on stainless steels utensils (1.1 $\log_{10}$) and polypropylene (0.9 $\log_{10}$), cutting boards, after 60 seconds holding time when surfaces were treated with ozonated water. In this study, increasing holding time resulted in an increased reduction of *L. innocua*. In our study, we observed increasing log reductions of *Listeria* with increasing contact times, but we used shorter contact times based on feedback for the feasibility of sanitation in industry. It is possible that ozonated water was not allowed enough time to inactivate more bacterial cells resulting in us not seeing any significant differences between the water and ozonated water sanitizer treatments.

We observed significant reductions of *L. innocua* on soiled stainless steel with ozonated water (Table 3.1) compared to reductions on clean surfaces. A possible factor influencing the reduction, in this case, could have been the pressure that was exerted on coupon surfaces due to the flow rate of ozonated water and water treatments. According to Pordesimo et al. (2002), higher wash water pressures enhance the physical removal of microbes and debris. Although the pressure of our ozonated water and water treatments was not measured, several studies have reported a significant reduction and/or dislodgment of bacteria due to pressure of water flow from tap or spray nozzle. (Uhlig et al., 2017; Yoder et al., 2010). This could be the reason for a higher reduction of *L. innocua* on soiled stainless-steel surfaces than on clean surfaces (P=0.01) as pressure from the flow rate of sanitation treatments could have resulted in bacteria and organic matter being dislodged and washed away.

Less reductions on soiled surfaces were expected because organic matter enhances bacterial attachment and protects from the biocidal effect of aqueous ozone (Gram et al., 2007; Korany et al., 2018). However, higher reductions were observed for *L. innocua* on soiled

stainless steel with both water and ozonated water treatments. Reductions of *L. innocua* on soiled polypropylene were also higher (3.21 $\log_{10}$) than on clean surfaces though not significantly different. This was an indication that organic matter did not have a protective effect on bacteria as has been reported in previous studies. For example, Korany et al., (2018) demonstrated the effect of organic matter on the reduction of single species of *L. monocytogenes* biofilm. In this study, the treatment with ozonated water for 1 minute at 4.0 ppm resulted in ca. 2.27 $\log_{10}$ reduction of single strain biofilm grown on polystyrene surfaces. However, when surfaces were conditioned with apple juice, and treated with ozonated water for the same exposure time, and concentration, log reduction was ca. 0.36 $\log_{10}$. Additionally, there were fewer reductions when milk was also used as organic matter. The reduced efficacy of ozonated water is a result of reactions with unsaturated organic compounds to produce unstable ozonoids that decompose rapidly (Staehelin & Hoigne, 1985). Meat and meat products contain unsaturated fatty acids that readily consume ozone leading to the production of hydrogen peroxide in some cases (Cobos & Díaz, 2015; Pryor et al., 1991). Meat soil systems with more greased surfaces have been shown to protect *Listeria* from the biocidal effects of sanitizers (Gram et al., 2007). Because we used a deli turkey that had a formulation of 0.17% fat content which is lean (USDA, 2019) it can be suggested that our meat soil system did not allow for a more greased or soiled surface resulting in similar or even higher reductions on soiled versus cleaned surfaces.

3.4. Conclusion

This study was aimed at evaluating the efficacy of ozonated water for the decontamination of *Listeria* on food contact surfaces. There were no significant differences in reductions attributed to ozonated water compared to tap water washing. Spraying applications may have influenced bacterial reduction from surfaces by dislodging rather than actual

inactivation. In addition, the soil system with deli turkey may have not provided sufficient soil (grease) to reduce the efficacy of ozonated water resulting in similar reductions on both soiled and clean surfaces. Overall, data suggest that cleaning may be effective at reducing transiently attached *Listeria* from FCS.

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Tables

Table 3. 1. Log reduction of *Listeria innocua* on stainless-steel coupons.

Listeria innocua on stainless steel (mean log CFU/cm^2 ± SEM)				
	Soiled		Clean	
Time (s)	Ozonated water	Water	Ozonated water	Water
15	1.99 ± 0.18^{Aa}	1.81 ± 0.46^{Aa}	1.48 ± 0.34^{Aab}	1.30 ± 0.19^{Ab}
30	2.22 ± 0.32^{Aa}	2.03 ± 0.33^{Aa}	1.71 ± 0.21^{Aa}	1.53 ± 0.11^{Aa}
45	2.61 ± 0.13^{Aa}	2.43 ± 0.29^{Aab}	2.10 ± 0.87^{Ab}	1.92 ± 0.40^{Ab}

Rows (lowercase) and columns (uppercase) with the same superscript are not significantly different at a 5% significance level.

Table 3. 2. Log reduction of *Listeria innocua* on polypropylene coupons.

Listeria innocua on Polypropylene (mean log CFU/cm^2 ± SEM)

	Soiled		Clean	
Time (s)	Ozonated water	Water	Ozonated water	Water
15	2.02 ± 0.66^{Aa}	2.32 ± 0.70^{Aa}	1.75 ± 0.67^{Aa}	2.04 ± 0.65^{Aa}
30	2.58 ± 0.47^{Aa}	2.87 ± 0.66^{Aa}	2.30 ± 0.87^{Aa}	2.60 ± 0.88^{Aa}
45	2.91 ± 0.60^{Aa}	3.21 ± 0.43^{Aa}	2.63 ± 1.44^{Aa}	2.93 ± 1.25^{Aa}

Rows (lowercase) and columns (uppercase) with the same superscript are not significantly different at a 5% significance level.

Table 3. 3. Log reduction of *Listeria monocytogenes* on soiled stainless steel and polypropylene coupons.

	Listeria monocytogenes (mean log CFU/cm^2 ± SEM)		
Surface	Time(s)	Ozonated water	Water
Polypropylene	15	2.28 ± 0.74^{Aa}	1.77 ± 0.32^{Aa}
	30	1.91 ± 0.02^{Aa}	1.91 ± 0.14^{Aa}
	45	2.31 ± 0.15^{Aa}	22.4 ± 0.15^{Aa}
Stainless Steel	15	1.04 ± 0.07^{Aa}	1.07 ± 0.15^{Aa}
	30	0.95 ± 0.02^{Aa}	0.91 ± 0.24^{Aa}
	45	1.19 ± 0.0^{Aa}	1.28 ± 0.09^{Aa}

Rows (lowercase) and columns (uppercase) with the same superscript are not significantly different at a 5% significance level.

CHAPTER 4: GENERAL CONCLUSION

The overall goal of this thesis was to investigate the prevalence and control of *Listeria monocytogenes* in food facilities. First, we determined the prevalence of *Listeria* spp and *Listeria monocytogenes* in small and very small food facilities in Nebraska. For this objective, we visited and collected environmental samples from three facilities in Nebraska. Samples were analyzed for *Listeria* spp and *Listeria monocytogenes*. *Listeria* spp was present in all facilities but *L. monocytogenes* was not detected in any of the facilities. There was variation in prevalence across facilities due to plant-specific operations and procedures. The highest frequency of *Listeria* positive samples came from floors and drains. Mobile nonfood contact surfaces were positive in some facilities demonstrating potential cross-contamination especially if the platforms were moved in and out of the main producing area. This study highlighted the importance of sanitation of sites like drains and floors as well as the control of movement as measures to control the contamination and spread of *Listeria* in these processing environments. We mapped the distribution of positive sites on a plan of each facility to facilitate the identification and to observe possible scenarios of cross-contamination. This study was important in that, it provided data that these facilities would use to start an environmental monitoring program. We are expecting to expand this study in the future to include more facilities that need assistance in complying with food safety regulations. For future studies, we aim to provide onsite training to facilities to collect their environmental samples. We also intend to expand environmental monitoring studies to pet food facilities and additional RTE food operations in Nebraska. Finally, we plan on evaluating and improving *Listeria* recovery from primary enrichments.

Next, we observed the efficacy of ozonated water for the decontamination of *Listeria* on soiled food contact surfaces. For this study, stainless steel and polypropylene coupons constructed to 10

x 10 cm were conditioned with organic matter made from deli turkey and inoculated with *L. monocytogenes* strain ATCC 19115. Other experiments were performed with *L. innocua* strain ATCC 33090 separately. In addition, clean coupons were also included for experiments with *L. innocua*. Inoculated surfaces were exposed to 10 ppm of ozonated water for 15, 30, and 45 seconds, respectively. Tap water was included as a treatment. There were no significant differences in reductions attributed to ozonated water compared to tap water washing. Spraying applications may have influenced bacterial reduction from surfaces by dislodging rather than actual inactivation. In addition, the soil system with deli turkey may have not provided sufficient soil (grease) to reduce the efficacy of ozonated water resulting in similar reductions on both soiled and clean surfaces. Overall, data suggest that cleaning may be effective at reducing transiently attached *Listeria* from FCS. A major limitation in this study was the instability of dissolved ozone which created some inconsistencies in data causing us to use two reps instead of three. More reps will be performed in the future. For future studies, we intend to evaluate longer exposure times and higher concentrations of dissolved ozone in water. Additionally, we plan on using different types of organic matter mimicking other food matrices. It is also our aim to assess the efficacy of ozonated water for decontamination of utensils at home and food service operations. Finally, we intend to evaluate the effect of ozonated water on persistent/biofilm *Listeria monocytogenes*