South Dakota State University

Open PRAIRIE: Open Public Research Access Institutional Repository and Information Exchange

Electronic Theses and Dissertations

2019

Assessing Listeria Risk during Different Stages of Ice Cream Manufacturing and Storage

Neha Singh
South Dakota State University

Follow this and additional works at: https://openprairie.sdstate.edu/etd

 Part of the Dairy Science Commons, Food Science Commons, and the Microbiology Commons

Recommended Citation

Singh, Neha, "Assessing Listeria Risk during Different Stages of Ice Cream Manufacturing and Storage" (2019). *Electronic Theses and Dissertations*. 3184.
https://openprairie.sdstate.edu/etd/3184

This Dissertation - Open Access is brought to you for free and open access by Open PRAIRIE: Open Public Research Access Institutional Repository and Information Exchange. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of Open PRAIRIE: Open Public Research Access Institutional Repository and Information Exchange. For more information, please contact michael.biondo@sdstate.edu.

ASSESSING *LISTERIA* RISK DURING DIFFERENT STAGES OF ICE CREAM MANUFACTURING AND STORAGE

BY

NEHA SINGH

A dissertation submitted in partial fulfilment of the requirements for the

Doctor of Philosophy

Major in Biological Sciences

Specialization in Dairy Science

South Dakota State University

2019

ASSESSING *LISTERIA* RISK DURING DIFFERENT STAGES OF ICE CREAM MANUFACTURING AND STORAGE

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy in Biological Science degree and is acceptable for meeting the thesis requirements for this degree. Acceptance of this does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Sanjeev K. Anand, Ph.D. Date

Dissertation Advisor

Vikram V. Mistry, Ph.D. Date

Head, Department of Dairy and Food Science

Dean, Graduate School Date

This dissertation is dedicated to my Family for

believing in me and for encouraging me

to pursue my dream of higher education,

and

to my Advisor for guiding and supporting me

throughout this journey.

ACKNOWLEDGEMENTS

I would like to express gratitude to my Major Advisor, Prof. (Dr.) Sanjeev Anand for his guidance, immense support, and for sharing his knowledge, expertise and experiences with me. He always encouraged me to think independently and try out newer ideas within the overall realms of my research project. In addition to the research, he also provided me opportunities to assist him in his teaching labs and serve as his research lab manager. All these experiences helped me develop many more skills including instructional and lab management roles, in addition to developing my critical thinking abilities. I achieved highest levels of learning excellence under his guidance, which resulted in many awards and honors that I received at the University and National levels (American Dairy Science Association). I feel fortunate to be his student and thank him for giving me wings of knowledge and learning.

I would like to express gratitude to my committee members, Dr. Vikram Mistry, Dr. Gemechis Djira, and Dr. Jessica Meendering for providing their valuable feedback from time to time and sharing their thoughts that proved very useful.

Graduate School Dean Doerner and Assistant Dean Lounsbery are acknowledged for their support, especially sponsoring me to the Midwestern Association of Graduate Schools 3 minutes thesis contest 2018, and Excellence in Gradate Teaching Assistant Contest 2019 at Michigan to represent SDSU.

I had the great opportunity to work closely with a leading US ice cream manufacturer. I would like to thank Brian Kraus, Director Food Safety and Regulatory Compliance, Wells Enterprise Inc., for his time and willingness to

share the industry resources. This collaborative effort helped me learn skills necessary under the commercial large processing operations and apply my research project learnings in real time.

My special thanks to the faculty and staff of the department for all their support throughout my stay. My fellow lab mates are acknowledged for their friendship and company during long days and sometimes nights doing complex and long experiments, and for their cooperation in helping me discharge my duties as the lab manager. All other friends on campus are acknowledged for providing a homely environment, away from home during my stay here.

I feel grateful to the Dairy and Food Science Department for providing a cordial and friendly environment, and the state of art infrastructure resources, which made my stay very comfortable, enjoyable, and productive. The help of Electrical Engineering department is acknowledged for the SEM and AFM work. I would also like to acknowledge the Midwest Dairy Foods Research Center, and the Agricultural Experiment Station of SDSU for sponsoring my assistantship and for funding this project.

My deepest venerations to Almighty for providing me the wisdom and strength to accomplish my dream of higher learning at the highest levels of excellence, and for making me who I am.

Neha

Feb 11, 2019

CONTENTS

ABBREVIATIONS

AFM: Atomic Force Microscopy

BHI: Brain Heart Infusion

BLEB: Buffered Listeria Enrichment Broth

CDC: Centers for Disease Control and Prevention

CFU: Colony forming units

FDA: Food and Drug Administration

HACCP: Hazard Analysis Critical Control Point

MOX: Modified Oxford

PBS: Phosphate Buffered Saline

PHE: Plate Heat Exchanger

PMO: Pasteurized Milk Ordinance

RLM: Rapid'LMono

SEM: Scanning Electron Microscopy

LIST OF TABLES

Chapter 2

Chapter 5

LIST OF FIGURES

Chapter 2

Chapter 3

Chapter 4

Chapter 5

ABSTRACT

ASSESSING *LISTERIA* RISK DURING DIFFERENT STAGES OF ICE CREAM MANUFACTURING AND STORAGE

NEHA SINGH

2019

Listeriosis is a life-threatening infection caused by foods contaminated with *Listeria monocytogenes*. Some of the major ice cream recalls in recent years reaffirm the ability of this food-borne pathogen to survive in diverse dairy processing environments and cause cross contamination. Inspection reports revealed certain lapses in implementing adequate hygienic practices for *Listeria* persistence in the processing environment, leading to cross contamination of ice cream. The higher levels of cross contamination of raw ice cream mix might result in random heat-injured cells when exposed to minimum heat treatment (69°C for 30 min). Evidence about the presence of injured cells in ice cream mix may thus prove useful to establish the overall *Listeria* risk, which was the aim of this study. Challenge studies were conducted to evaluate the dose dependent presence of heat-injured cells of *Listeria*. Ice cream mix formulations of 4 different types (36, 40, 42, and 45% total solids) were inoculated at 2.0, 3.0, and 4.0 log cfu/g levels of *Listeria innocua* (an established surrogate). The dose levels were selected based on a likely cross contamination on the raw side from environmental *Listeria*, especially due to their resident nature and growth in harborage sites. The samples were exposed to minimum heat treatment (69°C for 30 min) and the survivors, including heat-injured cells, were enumerated

using standard protocols. A binary logistic regression model was fitted for evaluating the severity of risk. The influence of total solids, water activity, and pH variability were also studied on *Listeria* survival. The enrichment protocol, using buffered *Listeria* enrichment broth, followed by plating on modified oxford (MOX) agar and Rapid L'mono (RLM) medium, revealed the random presence of heat-injured cells in buffered *Listeria* enrichment broth, only at the highest dose level of 4+logs. Any potential risk from heat-injured cells was thus limited only to the highest levels of cross contamination, irrespective of the type of the mix. Significantly, none of the pasteurized ice cream mix samples supported the recovery of any heat-injured cells of *Listeria* during 72h holding at 7°C, even at the highest dose level of 4+logs, under the conditions of experimentation. Based on this part of the study, the level of cross contamination (dose) emerged as a predictor of the potential presence of heat-injured cells of *Listeria* when exposed to minimum pasteurization treatment.

In the second phase of the study, we proposed a novel concept involving the possible protective role of air cells in the random presence of heat-injured cells. Challenge studies were conducted by inoculating ice cream mix samples (42% total solids) with *L. innocua* at a mean spiking level of log 4.0 cfu/g. The inoculated samples were subjected to minimum batch pasteurization treatment at 69°C for 30 min, and any heat-injured cells were enumerated using buffered *Listeria* enrichment broth (BLEB), followed by plating on MOX and RLM agar. Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) were conducted on the air-dried, spiked ice cream mix samples, at pre- and post-

thermal treatment stages, which showed the presence of large air cells with some entrapped cells of *Listeria*. In the post heat- treated mix, SEM and AFM micrographs showed entrapped cells only within the larger air cells, whereas the mix matrix, did not show the presence of any *Listeria* cells. These observations thus suggested that the *Listeria* cells entrapped within the larger air cells might not have received adequate thermal effect and resulted in their random presence as heat-injured cells, as detected by enrichment protocol.

In the third phase of the study we compared the effect of any temperature abuse of ice cream with the exposure to simulated gastro-intestinal fluids on the recovery potential of any carried over injured cells. Ice cream mix samples with injured cells were used in this study. Direct plating on *Listeria* selective agars was used for enumerating intact cells, while the heat-injured cells were recovered using BLEB, prior to enumerating on selective media. Although no intact cells were observed, the enrichment protocol revealed the random presence of heat-injured cells at the post-pasteurization stages of processing. Freezing and hardening steps did not appear to have any further detrimental impact on heat-injured cells, carried over from the pasteurized ice cream mix. The temperature abuse conditions, evaluated in the current study, although led to pudding consistency of ice cream, did not support the recovery of heat-injured cells. This thus implies that the post-pasteurization contamination with intact *Listeria* might pose a greater risk than any carried over injured cells. Similarly, such injured cells did not show any recovery in the simulated gastro-intestinal fluids tested under in vitro conditions. In the case of spiked intact cells, no detrimental effect of

freezing and hardening steps was observed. Results from this study emphasize a need to design stage-specific critical control points to prevent any potential *Listeria* outbreaks.

INTRODUCTION

Listeria is generally secreted in milk of normal as well as cows suffering from mastitis and has the potential to survive the manufacturing and ripening of many different cheeses. According to U.S. Department of health and human services Food and Drug Administration, *Listeria* has become a great challenge for the food industry as it can withstand the cold environment and can easily be found in moist conditions both of which commonly exist in a food processing plant. The widespread nature of *Listeria monocytogenes* demands systematic approach towards food safety and proper sanitation during ice cream manufacture. The bacterium also exhibits general stress response and biofilm formation under unfavorable environmental conditions that makes it more difficult to control in food processing plants as these make bacteria more resistant and tolerant to physical and chemical treatments. The principal factors that influences the survival and growth of *Listeria monocytogenes* in food are processing techniques, intrinsic (e.g. pH, water activity) as well as extrinsic features of the product (e.g. storage temperature). The overall incidence of *Listeria monocytogenes* in raw milk appears to be 2.2%, excluding Spain. Therefore, it is necessary to consider raw milk which comes to dairy plant for processing as a source of *listeria* contamination by the dairy processors. According to the process guidelines of the U.S. Food and Drug Administration, the minimum heat treatment of 71.7°C for 15 is sufficient to kill *Listeria monocytogenes* in milk. However, several studies have revealed that *Listeria* is an intracellular parasite, which can be found within the white blood cells in *Listeria monocytogenes*

contaminated milk and can survive pasteurization. Despite the implementation of a strong quality control program in the manufacturing units, *Listeria* has often been found to defy a logical pattern of persistence in the dairy processing facilities. Thus, it has become mandatory to identify the risk factors involved during ice cream manufacturing that directly or indirectly influences the cross contamination of ice cream with *Listeria* during the manufacturing process. These risk factors can help to generate more robust critical control points and can further be used to build a strong *Listeria* prevention program.

A typical ice cream manufacturing process is consisted of three basic steps (1) Operations that involves raw unpasteurized milk, (2) Operations involving pasteurized mix and (3) Operations involving frozen ice cream. The process starts with blending, where milk is blended with other dry ingredients which are then pasteurized (69 °C for 30 minutes or at 80°C for 25 seconds) either by batch pasteurizers or by continuous pasteurization method to destroy pathogenic microorganisms present in the ice cream mix. The mix is further homogenized at the pasteurization temperature to reduce fat globule size present in milk. After pasteurization and homogenization, new ingredients can be added to the mix. Although, these ingredients do not support microbial growth because of high sugar content, low pH and low water activity but these can allow the organism to survive if it had gained entry in the mix because of higher cross contamination leading to inadequate pasteurization effect. There is always a possibility of cross contamination if *Listeria* is present in higher numbers on the raw side along with the other background microflora, and if the product happened

to be contaminated at the post pasteurization stage, *Listeria* will survive through the later stages of ice cream processing and storage, as it can tolerate cold temperatures. Thus, it warrants application of Hazard Analysis Critical Control Point (HACCP) system in the ice cream manufacturing at every production step to avoid, reduce or minimize potential hazards and ensure products of good quality and safety. As *Listeria* does not withstand proper pasteurization treatment, however, improper pasteurization, and mishandling at multiple points along the food production chain might become the root cause of an outbreak. It has also been demonstrated that the contamination with *Listeria monocytogenes* might also occur at post processing steps, from equipment, pathogen prone areas, and due to plant hygienic conditions.

Listeria can be introduced into a processing plant in a number of ways; it can blend in with the ingredients, can come along with the processing aids and can be transmitted through plant environment via clothing and body, people's shoes, dust, equipment, packaging and inadequate cleaning and sanitation. The bacteria may also form a biofilm that is more difficult to eradicate. The fundamental quality assurance system in a food processing industry includes HACCP that start with raw material collection and continue through to finished product and handling. Independent variables such as pH, water activity, total solids, and overrun percent in the ice cream manufacturing process can also be standardized to optimize operating conditions for *Listeria* inhibition through response surface models. Based on the inherent variation of the parameters in the process and their effect on *Listeria*, tolerance limits for each parameter can

be set to achieve the desired outcome. In view of the above, the focus of this investigation was to understand *Listeria* survival at different stages of ice cream manufacturing and to identify the factors associated with its survival potential due to cellular injury.

CHAPTER 1

LITERATURE REVIEW

NEHA

Listeria monocytogenes is an intracellular facultative pathogen, which can grow at a pH range of 4.7 to 9.2 (Petran et al.,1989 and Phan-Thanh et al., 1998), refrigeration temperatures of -0.5 to 9.3°C (Walker et al., 1990) and salt concentrations of as high as 10% (McClure et al.,1989). Eating food contaminated with *Listeria* can lead to listeriosis, a severe infection associated with high rate of hospitalization and fatalities (Mead et al., 1999). Immunocompromised people, pregnant women and their newborns, and geriatrics (Doyle 1985; Silliker 1986) have been found to be at greater risk for developing Listeriosis with a death rate of 30% in the elderly and newborns (Doyle 1985). The primary symptoms of listeriosis in humans are meningitis, abortion, and perinatal septicemia (Silliker 1986). If left untreated, the infection further develops and leads to death as a result of meningitis. Although statistics reports depict a decline in the incidences of listeriosis over the past few years (CDC, 2005) but the outbreaks, and recalls due to contaminated food products still continues. Keeping in mind the severity of risk associated with this pathogen, FDA has established a zero-tolerance policy for listeria monocytogenes in ready to eat foods.

Food manufacturing process typically involves several manufacturing steps and use of different temperature conditions. These manufacturing and

temperature conditions also provide favorable growth environments to the pathogen hence, the presence of *listeria* in a typical food processing plant is not uncommon. In addition, the ability of *Listeria* to withstand extreme environmental conditions makes it difficult for the food processors to completely eradicate its presence within the manufacturing environments therefore, it increases the chances of cross contamination of various food products. Since, the infection is associated with longer incubation time, at times it becomes difficult to even trace the contaminated food product. Hence, the repeated presence of *listeria* in several environments and its ability to tolerate several extreme conditions poses a greater challenge to the food manufacturing industries. Some reports have also mentioned the association of the organism with the retail environments (Sauders et al., 2004 and Sauders et al., 2009). Several molecular subtyping studies have revealed the persistence of specific *listeria* strains for years within food processing environments (Miettinen et al., 1999, Norton et al., 2001, Lundén et al., 2003, Lopez et al., 2008). These persistent subtypes have occasionally been reported to cross contaminate the food products and have led to several outbreaks of *Listeria* (Ferreira et al., 2014).

The following sections of this chapter provides more in-depth information about *listeria* and its significance in the food manufacturing environments, as compiled from the available literature sources.

Foodborne Disease Outbreaks and Recent Recalls in the USA

Listeria monocytogenes is a food borne pathogen that is mostly found to be associated with recall of frozen products, and in recent years, even ice cream has been implicated. According to Centers for Disease Control and Prevention, between years 2007-2014, 31 pathogens led to 37.2 million illnesses, of which 9.4 million were foodborne. These pathogens led to 2,612 deaths, out of which 19% deaths were due to *L. monocytogenes*. The infectious dose of *L. monocytogenes*, which is to be ingested in order to cause a disease in humans, is mainly strain and host dependent, and not yet certain. Scallan et al (2011) in a report mentioned that the estimation of foodborne illness can be efficiently used to improvise food safety policies, which further can help in designing newer intervention strategies. The data provided in their report from active and passive surveillance as well as from other available sources revealed that each year, 31 pathogens lead to 9.4 million foodborne illnesses, 55,961 cases of hospitalization and a total of 1,351 deaths in the United States. Out of which 19% deaths were due to *Listeria* monocytogenes.

In Massachusetts, a *listeria* related outbreak in 1983 infected 49 people and led to fourteen deaths (Silliker 1986). The CDC investigation in this case revealed the consumption of a specific pasteurized whole or 2% milk (Pearson and Marth 1990; Doyle 1985) which further was linked to several farms where listeriosis had infected the dairy cows.

An outbreak occurred in 2015, because of consuming milk shakes made from ice cream, indicated that highly susceptible persons are likely to develop the

infection even after consuming low levels of contaminated food products (Pouillot et al., 2016). They reported that out of 2,320 samples, 4 samples had a concentration of >100 CFU/g. The average level of contamination in this study, was found to be low as compared to the several other *listeria* related outbreak, (Linnaen et al., 1988, Johensen et al., 2010, Pérez-Trallero et al., 2014, Heiman et al, 2016, Pouillot et al., 2016).

Overall, in the case of ice cream, according to Centers for Disease Control and Prevention (CDC), there were nine *Listeria* related ice cream recalls during 2014-2015. The Blue Bell incidence in March 2015 led to 10 hospitalizations and 3 deaths. Later in April 2015, Jeni's splendid ice cream tested positive for *Listeria* and recalled all the products. During sampling of two dairy processing plants to evaluate the traditional and molecular methods for *Listeria* detection, it was observed that the molecular method depicted higher *Listeria* contamination within the processing environment. Some equipment within the dairy processing environment found to have been contaminated with 10^{-4}- 10^{-5} cfu of *Listeria monocytogenes* (Alessandria et al., 2010). More recently in 2017-18, a voluntary recall issued by Filedbrook Foods Corporation of all the orange cream bars and chocolate- coated vanilla ice cream bars indicated the possibility of *listeria* contamination in the finished products line.

Listeria monocytogenes **as a Resilient Food Borne Pathogen**

The gram-positive *Listeria* species are ubiquitously present in nature. These are, non-spore forming rod shaped bacteria (Silliker 1986) which can exhibit growth at temperature ranges of 3-45°C, with an optimum of 30-37°C, pH

of 4.4-9.6, and salt concentrations of 10% (Pearson and Marth 1990; Meyer and Donnelly 1992). According to Lovett and Twedt (1988), *Listeria* species have even survived in salt concentration of 30.5% at 40°C for 100 days. These characteristics help *listeria* to survive in the extreme conditions and makes it difficult to control as compared to its other competitive counterparts (Meyer and Donnelly 1992). *Listeria* can easily be isolated from raw as well as pasteurized milk, ice cream, soft cheeses, vegetables etc. (Lovett, 1989). Its incidence as a food borne pathogen is of great importance due its ability to grow under refrigeration temperatures (4°C). In contrast, the organism is also found to be quite heat tolerant, as mentioned by Boyle et al.,1990 where they inoculated 80% lean ground beef with 8.08 log CFU/g *Listeria monocytogenes* and noted 0.2-0.9, 1.6-3.4 and 4.4-6.1 log reductions when heated at 50, 60 or 65°C. Frequent isolation of *Listeria monocytogenes* from frozen food has been noted by Cotton and White (1992). Azizoglu et al., (2009) investigated the impact of growth temperature (4, 25, and 37°C) on the cryotolerance of several strains of *L. monocytogenes* isolated from several food processing environments. It was found that the cells which were grown at 37°C had greater cryotolerance. The cryotolerance was decreased when these cells were grown at 4 or 25°C, depending on the strain variability. Hence, the findings from this study suggested that growth at 37°C is of importance and is necessary for expression of certain virulence determinants of *L. monocytogenes*, which further protects the organism against freeze-thaw stress.

Listeria monocytogenes, Environmental Persistence and Cross Contamination

Although, *Listeria* is ubiquitous and can be isolated from various sources, but it has also been reported to be associated with the dairy farm environment. In the context of raw milk contamination, the incidence of *L. monocytogenes* on cattle and small ruminant farms was reported to be 22.2 and 16.8%, respectively, in New York and adjacent states (Nightingale et al., 2004). Feeding cattle with poor quality silage, lack of following good practices during milking and insufficient cleaning of cows enhanced possibility of raw milk contamination with *L. monocytogenes* (Sanaa et al.,1993; Hunt et al., 2012). During a regular sampling of bulk raw milk on a dairy farm, it was found that the pathogenic *L. monocytogenes* was present at numbers less than 100 cfu/mL. The molecular testing depicted the possibility of direct excretion of organism into the milk. One cow, out of 180, had *L. monocytogenes* excretion at a level of 280 cfu/mL, suggesting risks associated with direct contamination (Hunt et al., 2012). On the other hand, sometimes, mastitis, encephalitis and abortions due to *Listeria* infection in cattle can also result in shedding of organisms in the milk (Fleming et al., 1985), which in turn lead to the cross contamination of milk by *Listeria*.

Listeria Survival as Injured Cells in Heat-Treated Products Such as Ice Cream

Upon exposure to various environmental stresses, microorganisms can experience structural as well as metabolic injuries. Some of the factors that may lead to bacterial injury includes high temperatures, freezing, freeze- drying, use

of preservatives, acidic environments or treatments like irradiations (Fung and Bosch 1975; Foegeding and Ray 1992; Meyer and Donnelly 1992). In addition to processing conditions, storage and handling of food products can also lead to generation of a stress response and further injury within microorganisms. A cell in the injured state loses some of its characteristic features (Busta 1978). For example, loss of salt tolerance ability has been reported in *Staphylococcus aureus* (Fung and Bosch 1975) and *Listeria monocytogenes* (Meyer and Donnelly 1992). The injured cells sometimes even fail to form colonies on a selective medium therefore requires a nonselective or a complex nutrient medium (Busta 1978; McDonald et al. 1983). The injured cells have also shown to have an extended lag phase compared to the uninjured cells (Busta 1978). Studies have indicated the degree of cell damage differs from organism to organism and is largely dependent on type of stress. The recovery of these injured cells is further dependent on the type of recovery media chosen for the analysis (Ray 1979).

Almost all injured cells have damaged permeability barrier due to which cells become susceptible to selective agents ultimately leading to structural injury. While on the other hand, cells may also undergo metabolic injury where the metabolic activities of the cell get affected (Ray 1979). The sublethal injury leads to cellular changes in the microorganisms and is subjected to the cell surface and cytoplasmic membranes.

In case of leakage of cellular material in the surroundings, the cells lose its ability to multiply in the usual manner unless restored (Ray 1979). One of the

important aspects associated with injured cells is their ability to repair upon exposure to suitable environments. Even though, a pathogen cannot cause an illness in the injured state but, upon returning of favorable conditions, these injured cells may repair themselves, and can regain pathogenicity (Foegedmg and Ray 1992). Hence, identification of sub lethally injured microorganisms, especially in the context of pathogens, is critical for the interpretations in a given food manufacturing and handling practices (Hoffmans et al., 1997).

Research work conducted in the direction of recovery of injured cells has indicated that the repair is directly related to the type of cellular changes that might have occurred as a result of injury (Busta 1976). The injury is reversible under suitable environments, the optimum time and temperature required for the repair is dependent on the type of stress the cell might have undergone.

The intracellular presence of some *Listeria* spp., within leukocytes, might result in their greater ability to survive the minimum pasteurization temperatures (Doyle et al., 1987). For instance, in Massachusetts, an inadequate filtering system in a plant failed to clarify the milk prior to pasteurization and introduced leukocytes into the system. This resulted in an inadequate pasteurization effect, hence, lead to an outbreak due to presence of intracellular lipid-shielded organisms (Bearns and Girard, 1958). In the same study, a post-pasteurization recovery of *Listeria* was demonstrated, when the sterile milk was inoculated with 5×10^4 cfu/mL or greater and heated at 61.7 °C for 35 minutes. These recovered cells later multiplied at room temperature, reaching to 10^8 cfu/mL, without any noticeable changes in the quality of milk. Similarly, a study conducted by Farber

et al., (1988) revealed the significance of large inoculum size and potential survival abilities of the organisms during pasteurization.

Glass and Doyle in 1989 were able to reduce *L. monocytogenes* counts (> *2* log CFU/g), by heating sausages to 51.7C for 8 h or 57.2C for 4 h, however, it did not completely eradicate the organism. Although after sub-lethal treatment, bacteria lose their ability to grow under normal conditions, these cells retain the ability to repair themselves when exposed to favorable growth environments.

Additionally, Besse (2002) in his review mentioned that it was not feasible to eliminate all bacteria with thermal treatment, as heating lead to a physiological stress within cells leaving them injured. These injured cells could repair themselves once the favorable conditions are back and provide a threat to the product quality (McMahon et al., 2000). Doyle et al., (1987) concluded that the *L. monocytogenes*, residing within polymorphonuclear leukocyte in the milk of *Listeria* - infected cows, could withstand minimum thermal treatment of 71.7°C for 15 seconds in a HTST pasteurizer. The level of background microflora in ice cream mix is also likely to affect the presence of heat-injured cells. In addition to the inoculum size, the physiological state in which the cells exist as well as their varied response to stress also influences the survival of *Listeria* (Pascual et al., 2001). The viscous foods and food constituents like proteins, carbohydrates also increase the bacterial resistance to freezing damage (Speck and Ray, 1977).

The listeriosis outbreak related detailed information on the prevalence of *L. monocytogenes* in food samples have been shown to provide data that can be a better predictor of *Listeria* risk. This is especially applicable to the products

such as ice cream that generally do not support the growth of the organism. In addition, *L. monocytogenes* populations can remain stable at freezing temperatures for extended periods of time (Dean et al., 1996, Gougouli et al., 2008, Palumbo and Williams., 1991); which would keep the *Listeria* levels in ice cream unchanged during retail or at consumer end.

Researchers have used techniques such as most-probable-number (MPN) to enumerate *Listeria monocytogenes* in commercial ice cream samples implicated in listeriosis outbreak. This helped to confirm *Listeria monocytogenes* in 99% of the samples and helped to further understand the risk for highly susceptible populations (Chen et al., 2016).

Listeria Survival in Frozen Products

Several physical and metabolic damages have been demonstrated to cause sub-lethal injury to *Listeria monocytogenes* under extended cold-storage conditions and nutrient poor environment (Dykes, 1999). On such example is freezing induced damage of cell wall and plasma membrane (El-Kest and Marth 1992). On the other hand, cellular protein damages were observed on exposure to heating and chlorine-based sanitizers (Bunduki et al. 1995). Dykes (1999) also reported visible shrinkage of the cytoplasm and slight cell wall damage by electron microscopy under different cold storage conditions.

Detection of Injured Cells of *Listeria*

The background microflora that co-enrich during the recovery process of pathogens greatly interferes with the efficient detection as well as recovery of *Listeria.*

In a study conducted by Ottesen et al., (2016) during enrichment, the bacterial taxa such as *Anoxybacillus, Geobacillus, Serratia, Pseudomonas, Erwinia*, and *Streptococcus* spp. were also found along with *Listeria monocytogenes*. The incidence of *L. monocytogenes* was greater at hour 0 as compared to 4, 8, and 12 h of testing and the parallel increase in background microflora indicated their co-enrichment.

Also, presence of stressed microorganisms has been reported in many foods, including frozen, dried, heated, fermented and in foods containing antimicrobials (Read 1979). It is important to be able to recover both normal and injured cells as the cells with microbial injury due to the cell damages have been reported to escape detection using traditional methods (Ray 1979). Such protocols would give opportunity for injured cells and establish the risk due to their potential to recover at the consumer end. McDonald et al. (1983) studied injured cells by measuring the differences in counts of a stressed microbial sample plated on a selective medium (only uninjured will grow) with that of a nonselective medium (both injured and uninjured will grow). Similar evidence was provided by Foegeding and Ray (1992) who reported a slow increase in numbers on both media, with repair being distinguished by increased numbers on the selective medium only. They also recommended some alternative methods for ensuring detection of injured cells where liquid medium was used to repair vegetative bacteria and involved incubating (usually at 37°C for 1 to 5 h or longer) the sample containing injured cells in a nonselective medium to support their repair. This was followed by MPN technique or direct plating for

enumeration. Similarly, some of the solid media can also support repair of injured cells by using both surface plate or pour plate techniques using at least 1 to 4 hours of incubation. Overlaying with selective agar can help diffuse nutrients into the nonselective layer thereby creating a more selective environment. The challenges however remain to detect, isolate, and resuscitate sub lethally injured *L. monocytogenes* from diverse food products using current methods of detection, which primarily focus on intact cells enumeration. This would result injured cells to escape detection, potentially recover and cause a consumer safety risk. On similar lines Hoffmans et al. (1997) voiced an opinion that the regulatory methods for foods should provide for the resuscitation and detection of stressed pathogens as well as indicator organisms to assess the risk in a more robust manner.

CHAPTER 2

EXPERIEMENTAL PROTOCOL STANDARDIZATION FOR DETERMINING *LISTERIA* RISK IN ICE CREAM

NEHA

Abstract

Eating food contaminated with bacterium *Listeria monocytogenes* can lead to listeriosis, a life-threatening infection which mostly affects pregnant women and their newborns, adults of 65 years or more, and immunocompromised people (CDC 2015). *L. monocytogenes* defies all the logical patterns of existence thereby, even after following rigorous risk assessment protocols, there have been several multistate outbreaks and recalls in the recent years. The frozen products are most vulnerable to *Listeria* outbreaks, as the organism can easily survive at lower temperature ranges, leading to its persistence in the dairy manufacturing environments. Several recent ice cream recalls (FDA 2018) indicated the environmental cross contamination of ice cream with *L. monocytogenes.* In view of this, the present study was conducted to determine the overall risk of *Listeria* in ice cream, in a comprehensive manner. Different stages of ice cream manufacturing were selected to understand the behavior of organism, and the role of product constituents in providing protection against thermal treatments. This chapter reports the preliminary standardization of experimental protocols involving selection of a thermal resistance surrogate of *L. monocytogenes*, cell harvesting and inoculation optimization, validation of thermal treatments, and enumeration and recovery protocols for detecting injured cells.

Ice cream mixes of four different total solid levels were obtained from a commercial ice cream manufacturer. The microbiological (Standard Plate Counts) and chemical parameters (pH and water activity) of the mixes were tested using standard protocols. Keeping in mind the pathogenicity associated with *L. monocytogenes,* a non-pathogenetic thermal processing surrogate *Listeria innocua* ATCC 33090 was selected for the challenge studies. As the process variables, the inoculum levels were standardized to achieve 2.0, 3.0 and 4.0 log cfu/grams of *L. innocua.* The influence of background microflora on the enumeration of Listeria was addressed using selective agar media, modified oxford agar (MOX) and rapid' *Listeria monocytogenes* agar (RLM) in the spiked ice cream mix samples. The second variable selected was the thermal treatment, for which three pasteurization temperatures (80°, 85° and 90°C for 25 seconds, and 69°C for 30 minutes) were tested under a batch process. These temperatures were selected based on the industrial operating limits and were evaluated for the presence for heat injured cells in the treated mix samples. In addition to direct plating, a selective enrichment protocol involving buffered listeria enrichment broth (BLEB) was used to enumerate heat-injured cells of *listeria*. The microscopic observation was conducted to observe any morphological changes in *Listeria* at pre and post pasteurization stages. The influence of co-variates on the total solids, water activity, and pH variability on the survivability of *Listeria* was also studied. The data obtained on variables and co-variates was used to conduct statistical analysis and develop binary models.

Based on the growth curve experiments, activated *L. innocua* was incubated for 6 hrs. to harvest cells in the mid exponential phase and the spiking standardization was achieved in 90 grams portions of ice cream mix samples. Direct plating on MOX and RLM did not reveal any *Listeria* survival at any of the total solid levels, even at the highest inoculation level (dose) of log 4.0+. However, the enrichment protocol revealed the random presence of injured cells only at the highest dose level of 4.0 logs, and the lowest heat treatment of 80°C for 25 seconds. The presence of injured cells showed a non-significant trend with the level of total solids. The observed thermal tolerance was also validated using a HTST pasteurizer. The MOX and RLM media proved useful in detecting and differentiating *Listeria,* in the presence of background microflora, however, some deceptive colonies did appear on these selective media, which needed further identified by MALDI-TOF. This information proved critical in preventing any false positive identification based on cultural aspects and colony morphologies. The microscopic observations showed the heat injured cells to be slightly thicker and swollen as compared to the intact cells. Statistical analysis revealed that total solids, water activity, and pH variability did not show any influence on *Listeria* survival pattern, leading to a quasi-complete separation, indicating dose and the thermal treatment as strong predictors of risk. The results presented in this study formed the basis of further studies as reported in the later chapters of this dissertation.

INTRODUCTION

Listeria is generally secreted in milk of normal as well as in cows suffering from mastitis and has the potential to survive the manufacturing and ripening of many different cheeses (FSAI, 2005). According to U.S. Department of health and human services Food and Drug Administration, *Listeria* has become a great challenge for the food industry as it can withstand the cold environment and can easily be found in moist conditions, both of which commonly exist in a food processing plant. The widespread nature of *L. monocytogenes* necessitates systematic approach towards food safety assessment and management, especially for the frozen product category including ice cream. The bacterium also exhibits general stress response and biofilm formation under unfavorable environmental conditions that makes it more difficult to control in food processing plants as these factors make *Listeria* more resistant and tolerant to physical and chemical treatments. The principal factors that influence the survival and growth of *L. monocytogenes* in foods are processing techniques, intrinsic as well as extrinsic features of the product (Buchanan et al., 2017). In view of the incidence of *Listeria* in raw milk it becomes imperative to consider raw side of dairy processing areas as a source of *Listeria* cross contamination (Farber and Peterkin, 1991). According to the processing guidelines recommended by the U.S. Food and Drug Administration (FDA), the minimum heat treatment of 71.7°C for 15 is sufficient to kill *L. monocytogenes* in milk. However, several studies have revealed that *Listeria* is an intracellular parasite, which can reside within the white blood cells in contaminated milk and can survive pasteurization (Doyle et

al., 987). Despite the implementation of a strong quality control program in the manufacturing plants, *Listeria* has always been found to violate a logical pattern of persistence in the dairy processing facilities. Thus, it has become critical to identify the risk factors involved during ice cream manufacturing that directly or indirectly influence the cross contamination of ice cream with *Listeria* during the process and may lead to their survival. A more comprehensive knowledge about these risk factors would help to generate more robust critical control points and build a strong *Listeria* prevention program.

A typical ice cream manufacturing process is consisted of three basic steps (1) Operations that involves raw unpasteurized milk, (2) Operations involving pasteurized mix and (3) Operations involving frozen ice cream as depicted in Figure 1 (Goff, 2018). The process starts with blending, where milk is blended with other dry ingredients that are then pasteurized (69 °C for 30 minutes or at 80°C for 25 seconds) either by batch pasteurizers or by continuous pasteurization method to destroy pathogenic microorganisms present in the ice cream mix. The mix is further homogenized at the pasteurization temperature to reduce fat globule size. Even after pasteurization and homogenization, some new ingredients can still be added to the mix, such as fruits, nuts or bulky flavorings such as candy or cookie pieces. Although, these ingredients do not support microbial growth because of high sugar content, low pH and low water activity, but these can allow the organism to survive if it had gained entry in the mix because of higher cross contamination. *Listeria* can be introduced into a processing plant in a number of ways; it can blend in with the ingredients, can

come along with the processing aids and can be transmitted through plant environment via clothing and body, people's shoes, dust, equipment, packaging and inadequate cleaning and sanitation. The bacteria may form a biofilm that is more difficult to eradicate. Although, it is difficult to always determine the source of *Listeria* contamination, a possibility of higher cross contamination on the raw side cannot be neglected, which might lead to inadequate thermal effect. The interactive effect of factors such as levels of contamination, role of background microflora, chemical parameters, and the effect of thermal treatments, on *Listeria* survival were not clearly known at the time of initiating this study in context to the ice cream manufacturing process. Although, several modeling programs such as PMP, Compbase are available online but these models provide the survival pattern of the pathogen in the broth environments and do not involve actual product matrix conditions. Hence, after considering all these situations, this study was designed to work around the first and important step of ice cream manufacturing i.e. on the raw side of the product manufacturing. The hypothesis was that if *Listeria* is present in higher numbers on the raw side along with the other background microflora, it might lead to the injured cells that may fail to appear on direct plating but would be revived during the enrichment process. This chapter reports the preliminary standardization of experimental protocols involving selection of a thermal resistance surrogate of *L. monocytogenes*, cell harvesting and inoculation optimization, validation of thermal treatments, and enumeration and recovery protocols for detecting injured cells.

MATERIALS AND METHODS

Sourcing and Basic Analysis of Ice Cream Mix Samples for Challenge Studies

Based on total solid levels, four different types of raw ice cream mixes (36, 40, 42 and 45%) were procured from a commercial ice cream manufacturer. The samples were packed in plastic containers of 2 gallons and transported under cold conditions. These samples were formulated and processed in the R&D section of the commercial dairy plant and were used in all our studies to be consistent with the ice cream mix characteristics.

Upon receiving, the ice cream mixes were tempered at 40°C and were analyzed for microbiological and chemical parameters. For standard plate counts, ice cream mix samples were plated on Brain Heart Infusion (BHI) agar (Oxoid, Thermo Scientific, UK) by following the standard methods (Wehr and Frank, 2004). Eleven grams portion of the respective sample was aseptically drawn and mixed with 99.0 mL of phosphate buffered saline (PBS), using a stomacher (Stomacher$^{@}$ 400 Circulator, Seward Laboratory System Inc., FL, USA) for 30 seconds to obtain the first dilution. Further serial dilutions were made, in phosphate buffer saline (PBS), and plated on BHI Agar. The plates were incubated at 37°C and the colonies were counted at 24 hours to obtain aerobic plate counts. The water activity of the samples was measured using aqua lab CX-2 system (Aqua Lab, Decagon Devices, Inc.), and the pH was measured using A321 pH meter (Orion Star^{TM} A321, Thermo Scientific).

Selection, Activation, and Identification of Mid Exponential Phase of Surrogate Organism (Listeria innocua ATCC 33090)

In order to conduct the challenge studies, pure culture of *Listeria innocua* ATCC 33090 was procured from American Type Culture Collection (Manassas, VA, USA). This is a nonpathogenic thermal processing surrogate of *L. monocytogenes,* which has previously been used in thermal resistance studies (Ryser and Marth, 1999, Kozempel et al., 2000, Li et al., 2013). The freeze-dried culture was reconstituted and grown as per instructions provided by the supplier. The activated and harvested cells were preserved in the form of glycerol stocks in the lab at -80°C. For growth curve experiments, an activated culture was streaked onto pre-solidified BHI agar plate and the plate was incubated overnight. A loopful of culture was then transferred into a sterile flask containing 250 mL of BHI broth. The freshly inoculated flask was incubated in a shaker incubator at 37°C and was plated on BHI agar following the standard dilution schemes at the interval of two hours for 12 hours. The plates were incubated at 37°C for 24 hrs. The colony forming units were counted and plotted against time to create the growth curve and the mid exponential phase for the *L. innocua* strain ATCC 33090, was derived to further use for challenge studies.

Standardization of Spiking Levels for Challenge Studies

For standardization experiments, the cells of *L. innocua,* preserved in glycerol stocks, were activated and sub cultured twice in BHI broth (Oxoid, Thermo Scientific, UK). The sub-cultured tube was incubated for 6 hours at 37°C, centrifuged (2000 x g for 15 min at 4°C), and washed twice with PBS to harvest

the cells. Appropriate cell suspensions (2+, 3+ and 4+ logs cfu/grams) were made in PBS and dilutions were plated on BHI agar to confirm the spiking levels. The spiking protocol was also validated using 90 grams portion of each type of ice cream mix. The validation of inoculation levels was done by plating 25 g of spiked ice cream mix sample by suspending in 225 mL of PBS followed by and mixing in a stomacher to obtain the first dilution. The mix was further diluted in a serial manner and plated by pour and spread plate techniques on an esculin based medium, Modified Oxford agar (MOX; Remel, San Diego, CA) containing containing 1% Moxlactam selective supplement**.** The plates were incubated at 37°C for 24 h, and were observed for typical *Listeria* colonies, which were small black with a black halo (Park et al., 2013) on MOX and white, with or without halo on RLM (Bio-Rad specification sheet). The counts were recorded as log cfu/grams.

Thermal Treatment (lab Pasteurization) of Spiked Ice Cream Mixes

The spiked ice cream mix samples (inoculated to obtain 2.0, 3.0, 4.0 cfu/g *L. innocua*) were individually batch pasteurized in a shaking water bath (Lab companion AAH44063U 17L reciprocal shaking water bath, Cole- Parmer, USA). Each dose level was thermally treated at three different temperatures (80°, 85° and 90°C) for a duration of 25 seconds each, under lab conditions. The time temperatures combinations were selected based on the range commonly practiced in ice cream industry. The bottles containing spiked ice cream mix samples were held in the shaking water bath, and the core temperature was monitored using a data logger (Fisherbrand, traceable waterproof K

thermometer, accuracy ±0.3°C). The treatment time was recorded as soon as the core temperature reached the desired temperature level. At the end of the treatment, the samples were rapidly cooled to 4°C in an ice jacketed basket and were used for further analysis. A corresponding batch time-temperature treatment of 69°C for 30 minutes was also evaluated to simulate the minimum pasteurization treatment (80°C for 25 sec).

Enumeration of Heat Injured Cells

Direct Plating of Pasteurized Mix Samples*.* For direct analysis of pasteurized ice cream mixes, 25 grams of the sample was suspended in a stomacher bag containing 225 mL of PBS. After mixing the sample, 1 mL from this suspension was direct plated on MOX and RLM to enumerate the uninjured (intact) *Listeria* cells that might have survived the heat treatment. This is based on the previous findings of Hansen and Knochel (2001) that direct plating on Oxford agar is likely supports the growth of uninjured populations. The plates were incubated at 37°C for 24 hours and were observed for typical *Listeria* colonies.

Recovery of Heat-Injured Cells using BAM Enrichment Protocol. Additionally, in order to enumerate any heat-injured cells of *Listeria,* an enrichment protocol was used, as recommended by USFDA (Bacteriological Analytical Manual, BAM, 2017). A 25 grams portion each of the lab pasteurized spiked sample was suspended in 225 mL of buffered *Listeria* Enrichment Broth (BLEB), and incubated for 4 hours at 30°C. *Listeria* selective enrichment supplements (Acriflavin HCl, Nalidixic acid and Cycloheximide) were added after

four hours and the samples were further incubated at 30°C. At 24 to 48 h intervals, BLEB enrichments were streaked on MOX agar and RLM to show the recovery of heat-injured cells. The results were recorded as positive or negative, based on the presence or absence of colonies on the selective media used for streaking, due to the recovery of heat-injured cells of *Listeria*.

Validation of Lab Pasteurization Treatment Using a Pilot Scale R&D Plate Heat Exchanger

The pilot scale validation of the dose response study was done using 36% (lowest) and 45% (highest) total solid levels of spiked ice cream mix samples using the minimum thermal treatment of 80°C for 25 seconds (that had resulted in the random presence of injured *Listeria* at stage 1 of the experimentation). Fifteen gallons of raw ice cream mixes (36 and 45%) were spiked with 4+ logs of *L. innocua.* Eleven grams of sample was aseptically drawn in a stomacher bag and was mixed with 99.0 mL of PBS to obtain the first dilution, which was serially diluted to plate on MOX agar to confirm the spiking levels of 4+ logs. The spiked ice cream mix samples were pasteurized at 80°C for a duration of 25 seconds each by using pilot scale R&D plate heat exchanger (HTST Plate Heat Exchanger, APV). The samples were collected at two points, start (175.9°F, holding time 25 seconds) and end (176.0°F, holding time 25 seconds) of pasteurization) and were analyzed further for direct plating on MOX plates as well as for the recovery in BLEB.

Microscopic Techniques for Studying Cellular Morphology of Intact and Injured Listeria

During the recovery experiments for detecting injured listeria using BLEB enrichment followed by selective plating on MOX and RLM, the typical *Listeria* colonies were gram stained and viewed under light microscope (oil immersion lens) to observe any changes in the morphology of cells. In a parallel experiment, intact *Listeria* cells were suspended in BHI broth and exposed to minimal thermal treatment. The samples were smeared onto the glass slides at pre and post thermal stages. The smears were air dried by using the method described by Hassan et al., (2010) and SEM analysis was done to view the intact (in spiked) as well as injured cells (in thermally treated samples) under the scanning electron microscope (Hitachi S-3400N, Hitachi America Ltd., Tarrytown, NY), located in the Daktronics Engineering Hall, South Dakota State University.). The slides were sputter coated with a 10-nm-thick layer of deposition of 99% gold and observed at 10 kV accelerating voltage by maintaining a distance of 10 mm from the slide.

General Statistical Analysis and Binary Logistic Regression

The data from the plate count results were subjected to analysis of variance (ANOVA) using SAS® 9.3 (SAS Institute Cary, NC, USA). The level of significance was set to 5%. For the challenge studies, three dose levels (log 2.0, 3.0, and 4.0) were tested for thermal resistance and presence of any heat injured cells of *Listeria* at different pasteurization temperatures (80,85 and 90°C). In order to model the risk, the standard binary logistic regression was applied. The

binary dependent variable included the absence of heat-injured cells, recorded as 0 (zero), and their presence (recovery) recorded as 1 (one).

During further study, using enrichment broth, the independent variables considered for the potential recovery of heat-injured cells were dose, temperature, water activity, and pH. Graphical descriptive analyses were performed to study the joint distributions of water activity and pH by total solids levels. Boxplots of water activity by total solids, and pH by total solids were constructed. At a later stage of the modeling process, penalized logistic regression was also used, due to the quasi-complete separation. Non-significant predictor variables were eliminated, and the water activity and pH data were used to generate boxplots for different total solid levels.

RESULTS AND DISCUSSION

Basic Analysis of Ice Cream Mix Samples for Challenge Studies

Four types of ice cream mixes having total levels ranging 36-45% were obtained from a commercial ice cream manufacturer. These were analyzed for background microflora, water activity and pH upon arrival. The data presented in Table 1 depicts the average standard plate counts of raw ice cream mixes, as received. The counts in samples ranged from log 4.5 to 5.97 with no specific order for the four total solid levels. Although we did not come across any other such reports on raw ice cream mix bacterial counts, even the processed ice cream has been shown to have range from 2- 5 logs (Campbell M., 2015). The counts were observed to be consistent within the three trials, conducted in the replicates of three, for each total solid level. This data helped us to establish a

base value for the background bacterial load in raw ice cream mix samples tested.

Chemical analysis of raw ice cream mix samples revealed that the water activity (aW) and pH of the ice cream mix samples varied within a narrow range despite of differences in the composition, as also reported by Gougouli et al., (2008) (Table 2). The aW and pH values were found in the typical range of 0.984-0.997 and 6.33-6.79, respectively, as generally encountered under commercial ice cream manufacturing conditions.

Developing Growth Curve of the Test Organism

Based on the colony counts and the duration of plating, a growth curve of *L. innocua* was obtained and the mid exponential phase was determined by plating the counts versus duration of incubation. Figure 2 depicts the growth curve of *L. innocua*, based on the graph plotted using the mean values from three trials. The data suggested the mid exponential phase for the test organism to be about 6 hrs. It may also be noted that the lag phase ended at 4 hrs., and the stationary phase began at about 8 hrs. In view of this, *L. innocua* was activated, sub cultured twice in BHI broth (Oxoid, Thermo Scientific, UK) and was incubated at 37°C for 6 hrs., at the time of spiking for each experiment while conducting the challenge studies. This gave us consistency in the number of *Listeria* cells being harvested for inoculating to ice cream mix samples before exposing to thermal treatments. This was an important part of the spiking level standardization, as we were not only focusing on the levels of inoculation to be 2.0, 3.0, and 4.0 logs for the respective experiments, but also wanted the cells to

be in comparable physiological state. It is well established that the mid-exponential growth phase results in the rapidly dividing cells, which are in the exponential phase of their growth. Such cells are mostly preferred in resistance studies and are considered very comparable in terms of the resistance being evaluated.

Standardization of Spiking Levels of the Test Organisms for Challenge Studies

Raw ice cream mix, along with its natural background microflora, was used for conducting the challenge studies to simulate the commercial manufacturing conditions. The hypothesis was that the cross contamination of raw ice cream mix with *Listeria* and their later presence as heat-injured cells may be influenced by the level of background microflora in the raw ice cream mix. As standardized in the experiments above, 6 hrs. old culture of *L. innocua* was centrifuged, washed twice with PBS and was diluted further, as per standardized protocol to achieve 2.0, 3.0 and 4.0 logs cfu/mL. Table 3 provides the mean counts obtained upon diluting the cultures as per standardization levels as achieved in PBS. We were successful in achieving the desired levels of cells in the inoculum for spiking purposes in a consistent manner. For spiking ice cream mixes, 90 mL of PBS was replaced with 90 grams of ice cream mixes. It may be observed form the data presented in Table 4 that the average pre-pasteurization spiking counts of *L. innocua* in ice cream mixes were comparable across all inoculation levels in all types of ice cream mixes ranging from 36-45% total solids. It may be observed that at 2+ spiking level the actual counts varied

between logs 2.33 to 2.54, at 3+ spiking level it varied between logs 3.41 to 3.48, and at 4+ spiking level it varied between logs 4.55 to 4.62. After completing the mid-exponential phase study for the test organism, this was the next important step in standardizing the challenge study protocol. The consistency of the inoculation process and obtaining reproducible counts in the ice cream mix, which itself is so viscous, was a big challenge. The reproducibility of spiking levels in ice cream mix thus helped to ensure the consistency of the testing protocols followed during the later experiments.

Colony Morphology on Selective Media

When the spiked samples were plated on *Listeria* specific medium, the colonies appeared on MOX appeared to be round, approximately 1.0mm in diameter, black in color and surrounded by a black zone (Figure 3A) and round, white, with or without halo on Rapid L'mono (RLM) agar medium (Figure 3B). This was consistent with the colony morphology as described by the media brochures (Modified Oxford Agar Base - Thermo Fisher Scientific, Rapid' *L.mono* medium). This experiment helped us validate the *Listeria* detection protocol using culture media. The selected colonies were sent for MALDI-TOF at the Veterinary Diagnostic Lab, SDSU for final confirmation. Based on these results, only MOX and RLM were used for detecting *Listeria* throughout the study.

The significance of this part of standardization is quite apparent, as the *Listeria* was needed to be isolated in the presence of background microflora of ice cream mix. Although, *Listeria* isolation protocol uses selective media, it was hypothesized that some other organisms might also show up on the selective

media and result in false positive tests. Results presented in Figures 3C and 3D provided a proof of concept for the hypothesis that occasionally, in addition to typical *L*isteria colonies, some deceptively similar colonies appeared on MOX and RLM. Hence, these colonies were isolated and purified by the single cell isolation culturing technique and were sent to MALDI-TOF for identification. As per the description of different colony morphologies presented in Table 5 and the MALDI-TOF identification, it was concluded that deceptively similar colonies could appear on MOX and RLM, even if the media are very selective for *Listeria* isolation. Similar results were also observed even while following the two steps; BLEB enrichment and selective media isolation protocol. This is very critical information in the isolation and identification of *Listeria* in the presence of background microflora. With this study, it was established that deceptively similar colonies of the surviving background microflora can appear on the selective media such as MOX and RLM, and hence may lead to false positive tests. As a standardized protocol, it would thus be important to conduct tests such as MALDI-TOF to confirm the presence of *Listeria,* even though the typical colony morphology on selective media may indicate it to be suspect *Listeria.* This thus proved to be another important finding in the standardization of our experimental protocols.

Thermal Treatment (Batch Pasteurization) of Spiked Ice Cream Mixes and Enumeration of Heat Injured Cells

In order to standardize the thermal treatment process for later experiments, preliminary studies were conducted using the spiked ice cream mix

samples and exposing them to different batch heat treatments at temperatures that are commonly attained in the commercial ice cream manufacturing plants. The effect of these thermal treatments (80°, 85°, and 90°C for 25 sec) and on the survival of *Listeria* was studied using standard isolation and identification protocols as outlined in the previous sections of this chapter. Thermally treated ice cream mix samples were direct plated, in addition to enrichment in BLEB. While the direct plating on selective media, helped us to establish the presence of intact cells (survivors), the BLEB enrichment protocol helped us to recover the heat-injured cells. The results obtained from direct plating on both MOX and RLM of ice cream samples, exposed to all three thermal treatments, did not result in the detection of any *Listeria* survivors, indicating the effectiveness of pasteurization in inactivating *Listeria,* even at the highest dosing (cross contamination) level of Log 4+. However, the enrichment protocol revealed the presence of injured cells that appeared randomly only in samples spiked at log 4+ levels and exposed to minimum batch pasteurization treatment at 80°C for 25 seconds batch pasteurization without any particular relationship with total solid levels (Table 6A and 6B). On the other hand, the lower dose levels of log 3+ and 2+ never showed any survivors on both direct plating and enrichment protocol. The thermal experiments were also repeated by using the batch process at 69°C for 30 minutes, and produced similar results to the equivalent process of 85°C for 25 sec. This part of study helped us to standardize the thermal treatment process for conducting the later experiments, which helped us to establish the cross contamination (dose) levels on the raw side of processing can serve as

predictors of the injured cells. This study is presented in Chapter 3 of this dissertation and has also been published (Neha et al., 2018).

Validation of Thermal Treatment using Pilot Scale R&D Plate Heat Exchanger

Since the above experiments were conducted using batch pasteurization, in a shaker water bath, the results were also validated by conducting a pilot scale trial using commercial R&D plate heat exchanger. The average log levels of spiked samples of pilot plant scale trial with 2+ and 4+ logs in 15 gallons of raw mix with *Listeria innocua* are presented in Table 7***.*** This helped us to standardize the inoculation levels in larger volumes of ice cream mix and thermally treat with HTST pasteurizer to indicate the validity of a pilot scale experimental trial. When the samples were direct plated after HTST pasteurization, the results did not detect any survivors on MOX in any sample, indicating the effectiveness of pasteurization treatment (80° C for 25 sec) in inactivating *Listeria innocua,* even at the highest dosing (cross contamination) level of Log 4+. This validated the batch pasteurization study as presented in the earlier section of this chapter. However, it was noted that the enrichment protocol using the selective medium protocol enumerated a few injured cells that appeared randomly in ice cream mix samples, spiked at log 4+ levels. As the enriched samples showed presence of injured cells, hence, it validated the lab scale study with regard to the detection of injured cells in samples spiked at 4+ log levels. This study thus helped to establish the comparable effectiveness of the batch treatment processes and facilitated conducting extensive experimentation at the lab scale.

Microscopic Techniques for Studying Cellular Morphology of Intact and Injured Listeria

In order to study any morphological changes in the heat-injured cells of *Listeria*, intact and injured *Listeria* cells were observed by gram stained smears, as well as, scanning electron microscope. It may be seen from Figures 4A, and 4B that the intact cells appeared short, regular rods whereas, the injured cells appeared slightly distorted rods (polymorphism). Similarly, the heat injured cells appeared to have ruffled surfaces as compared to the intact (uninjured cells, pre-heat treatment) under SEM, Figures 5A and 5B. This observation was similar to a previous report, where the ultrastructure analysis of heat-injured cells indicated cytoplasmic clearance and swollen cell surfaces as an impact of heat injury, while there was no leakage of cellular components observed (Novak and Juneja, 2001). Such injured cells might recover on restoration of the adequate growth conditions such as temperature and composition of the product (Mackey et.al., 1994) and create a risk for an outbreak. Based on the microscopic observations on the intact and injured cell morphologies, later studies were planned, where microscopy was incorporated as part of the evaluation protocol.

Developing Preliminary Binary Logistic Regression Modelling for Listeria Risk Assessment

Binary logistic regression was applied to evaluate the impact of dose and total solid levels on the survival of *Listeri*a at each of the pasteurization temperature. The model resulted in quasi-complete separation, which indicated dose as a strong predictor of risk due to injured cells of *Listeria*. A non-significant

trend was observed for injured cells recovery with the level of total solids. Figure 6A depicts the interaction of total solids and pH where the boxplots indicate a clear separation across different total solid levels. Figure 6B provides the boxplot interactions of total solids and water activity. It may be seen that with the increase in total solid levels, the water activity decreases across the total solid levels. The information on pH and water activity proved useful while evaluating the role of these two variables as covariates for developing regression models on the risk from the survival of heat-injured cells of *Listeria* in the cross-contaminated ice cream mixes.

CONCLUSIONS

This part of the study helped in standardizing experimental protocols for studying the *Listeria* risk in ice cream. For obtaining the exponentially growing cells, a 6-hour incubation period was derived based on the growth curve experiment. While evaluating the thermal process, it was found out that a minimum pasteurization temperature of 80°C for 25 seconds or corresponding 69°C for 30 min, under batch treatment conditions, resulted in random presence at the highest level of inoculation. It was also observed that the background microflora can appear on selective mediums in the form of deceptive colonies hence, more specific approach such as MALDI-TOF should be used for further confirmation of *Listeria*. This study forms the basis of further experimentation using the highest dose levels (4.0 cfu/g) in demonstrating the random presence of *Listeria* through different stages of ice cream manufacturing as discussed in later part of this dissertation.

Chapter 2 Tables and Figures

Table 1. Average standard plate counts (Log_{10} CFU/g) of raw ice cream mix samples as received form the industry.

Total Solids	Raw Ice Cream Mix Mean ±SE
36%	4.50 ±0.09
40%	5.78±0.03
42%	5.97±0.01
45%	4.95±0.02

n=9

Table 2. Water activity and pH values of the samples.

Sample	Type	Water Activity	pH
36%	Raw	0.995	6.33
40%	Raw	0.983	6.62
42%	Raw	0.974	6.79
45%	Raw	0.990	6.67

n= 9

Table 3. Standardization of *Listeria innocua* spiking levels for challenge studies in PBS.

Culture	Diluent	PBS	Mean± SE Log_{10} CFU/g
50 µL	50 mL PBS	90 mL	4.28± 0.004
5 µL	50 mL PBS	90 mL	3.42 ± 0.003
5 µL	500 mL PBS	90 mL	2.78 ± 0.016

n= 9

Table 4. Average of *Listeria innocua* spiking levels achieved in mix samples (Log_{10} CFU/g) for challenge studies.

Total Solids	Raw Ice Cream Mix Mean ±SE Lab Trial		
36%	2.52 ± 0.01	3.41 ± 0.03	4.62 ±0.04
40%	2.33 ± 0.00	3.48 ± 0.07	4.59 ±0.09
42%	2.54 ± 0.01	3.47 ± 0.06	4.57 ±0.12
45%	2.53 ± 0.07	3.47 ± 0.07	4.55 ±0.10

n=9

Table 5. Colony morphology of typical Listeria and some other deceptively similar background micro flora on MOX and RLM.

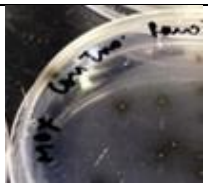
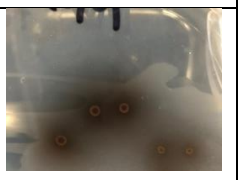
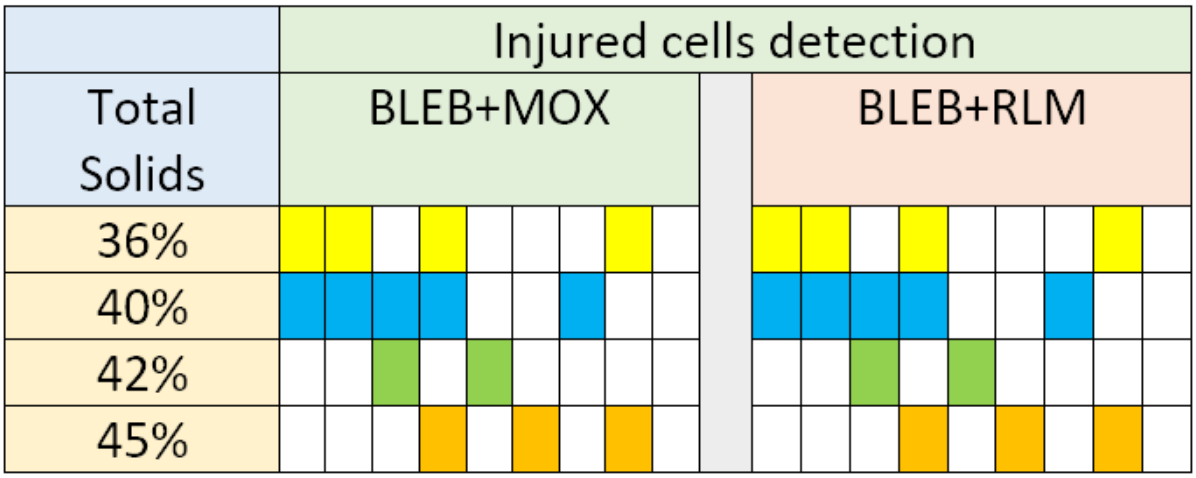
Colony Morphology	**MALDI-TOF**	**Photo**
Small black colonies with a black halo on MOX	*Listeria innocua*	
Small white, with or without halo on RLM	*Listeria innocua*	
Small black colonies without black halo on MOX	*Microbacterium paraoxydans*	
Small white, with or without halo on RLM	*Microbacterium paraoxydans*	
Dotted whitish with halo on MOX	*Bacillus licheniformis*	
Grey, pigmented and irregular	*Bacillus licheniformis*	

Table 6. Enrichment protocol results of the recovery of injured cells of *Listeria* in the lab pasteurized spiked ice cream mix samples (spiked at log 4+).



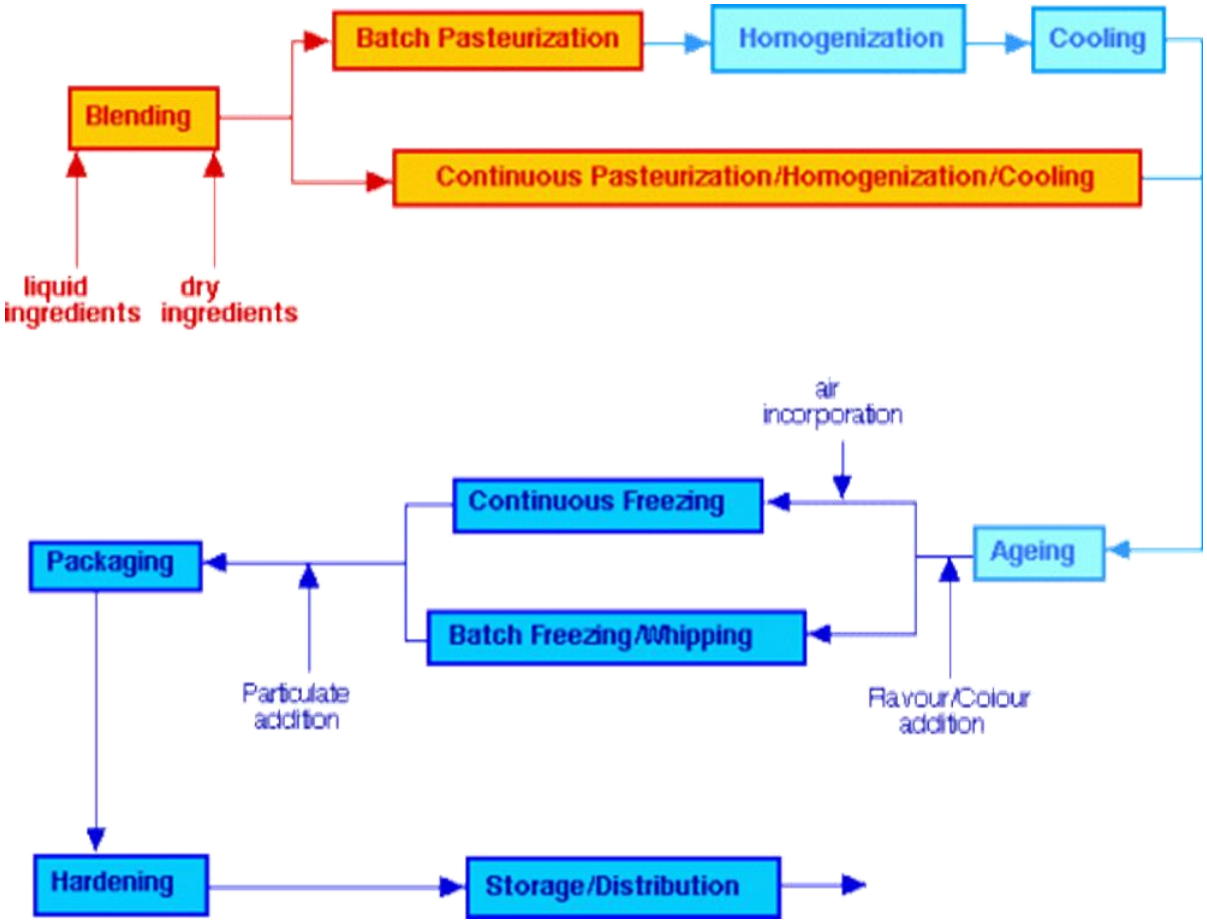
Based on three trials, the color squares show positive results irrespective of the total solid levels

Table 7. Average log levels of spiked samples of pilot plant scale trial with 2+ and 4+ log cfu/ g.

Total Solids	Raw Ice Cream Mix (Mean ±SE)	
		Pilot Scale Trial
36%	2.52 ± 0.01	4.74 ± 0.23
45%	2.53 ± 0.07	4.95 ± 0.30

n=3

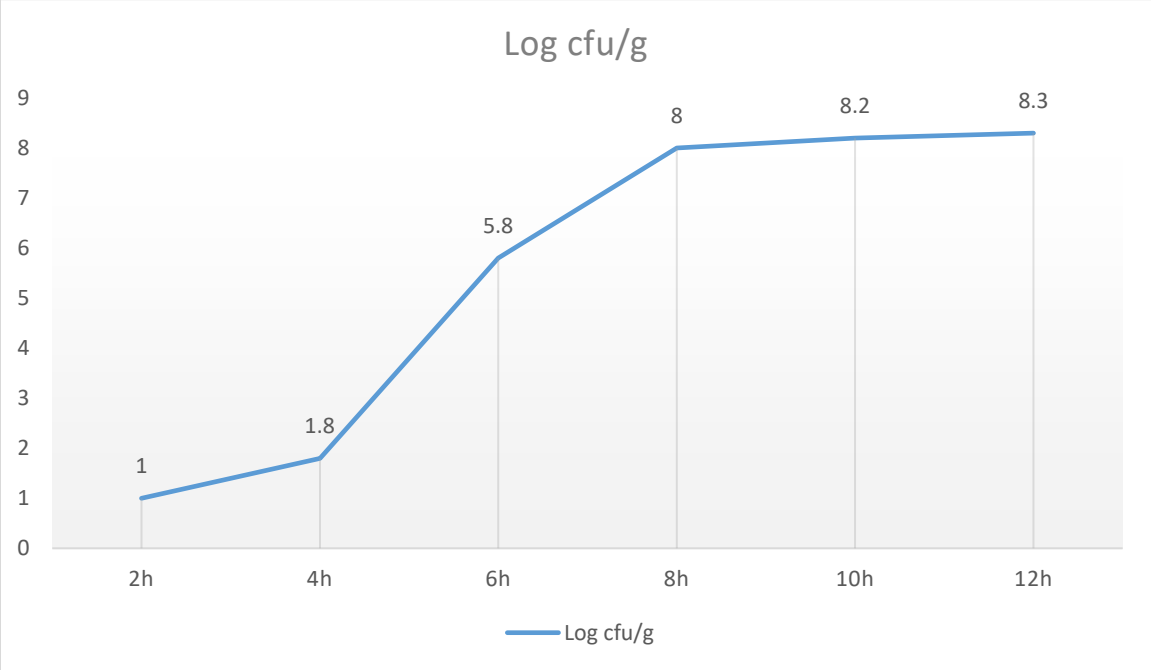
Figure 1. Flow Chart of the general Ice cream manufacturing process**.**



Sourced from: The Ice cream ebook, Univeristy of Guleph.

https://www.uoguelph.ca/foodscience/book-page/ice-cream-manufacture

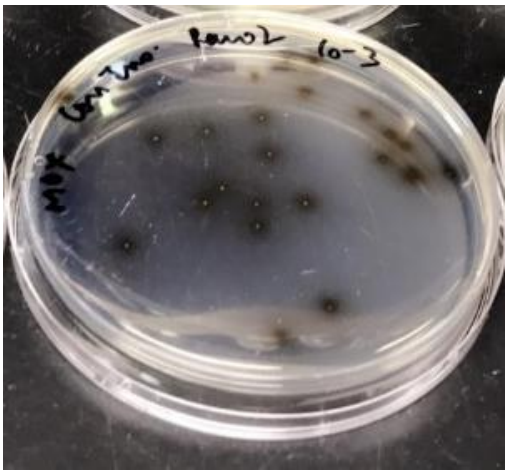
Figure 2. Growth curve of *Listeria innocua* at 37°C incubation in Brain Heart Infusion broth.



n=*3*

Figure 3A and 3B. The appearance of *Listeria innocua* on MOX agar medium and RLM medium.

3A



3B

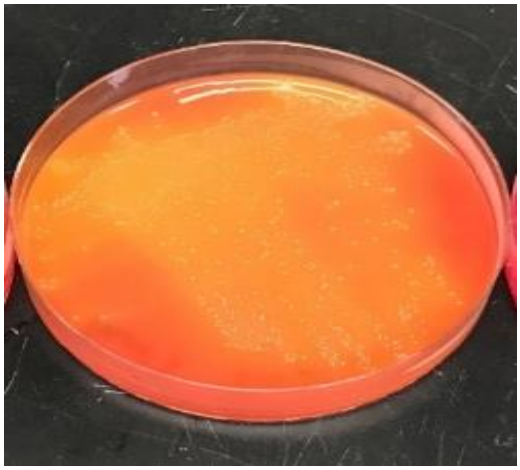


Figure 3C and 3D. The appearance of background microflora on MOX agar medium and RLM medium.

3C

3D

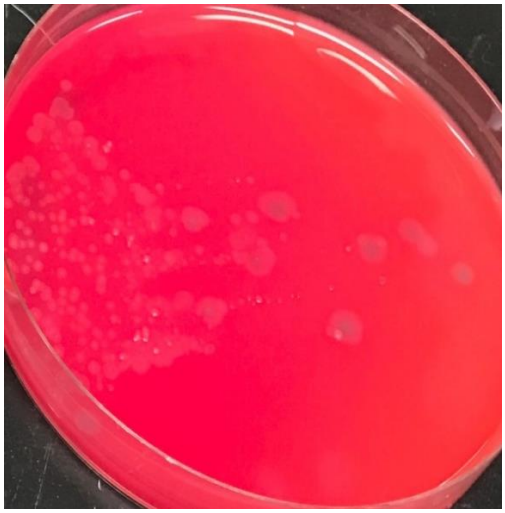
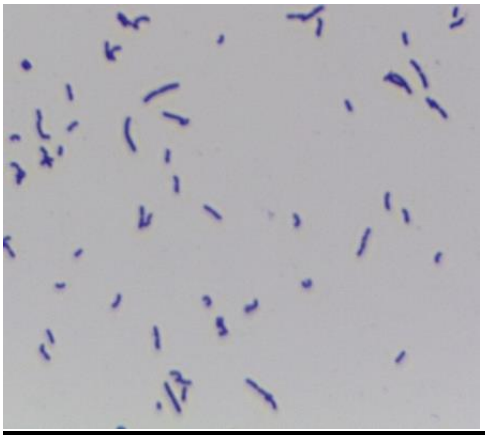


Figure 4A and 4B. Microscopic view of gram stained intact *Listeria* cells versus recovered *Listeria* cells on MOX medium after BLEB enrichment. The recovered *Listeria* depicting a potential polymorphism.

4A



4B

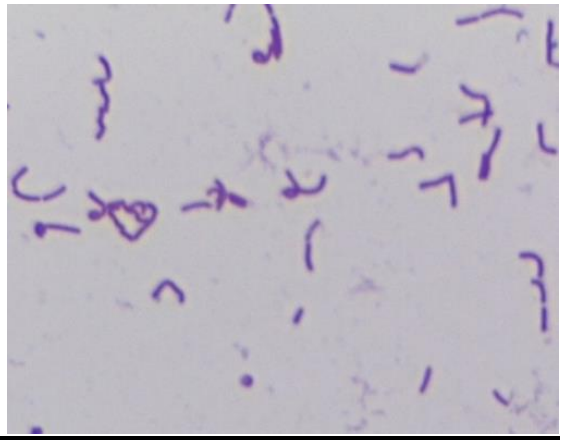
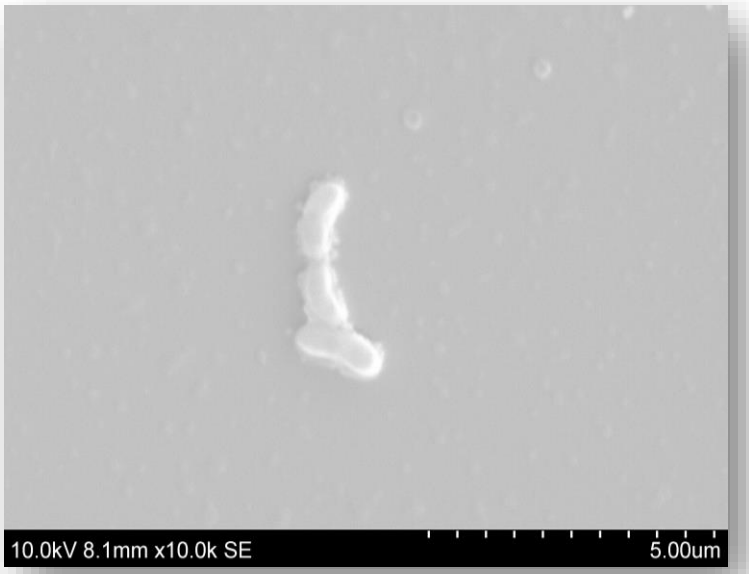


Figure 5A and 5B. Scanning Electron Microscopic images of *Listeria innocua in BHI broth.* (Magnification: 10K); Intact cells SEM image (A), and Thermally-treated cells (B).

5A



5B

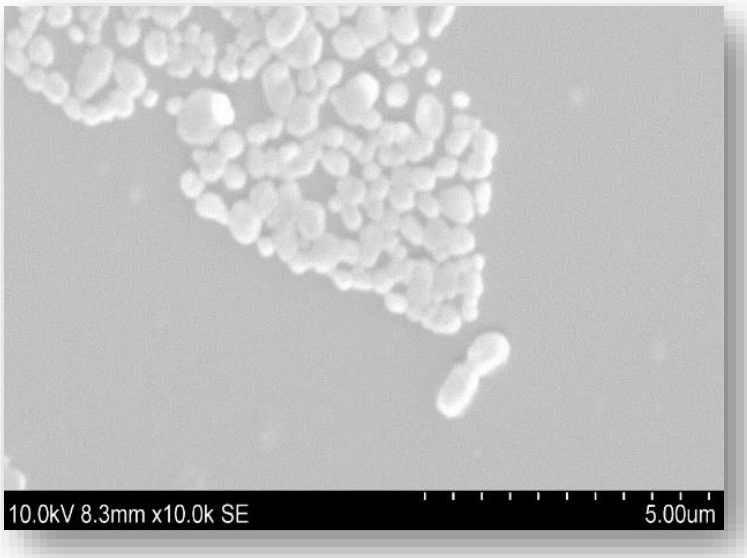


Figure 6A. Boxplot depicting interactions of total solids vs pH.

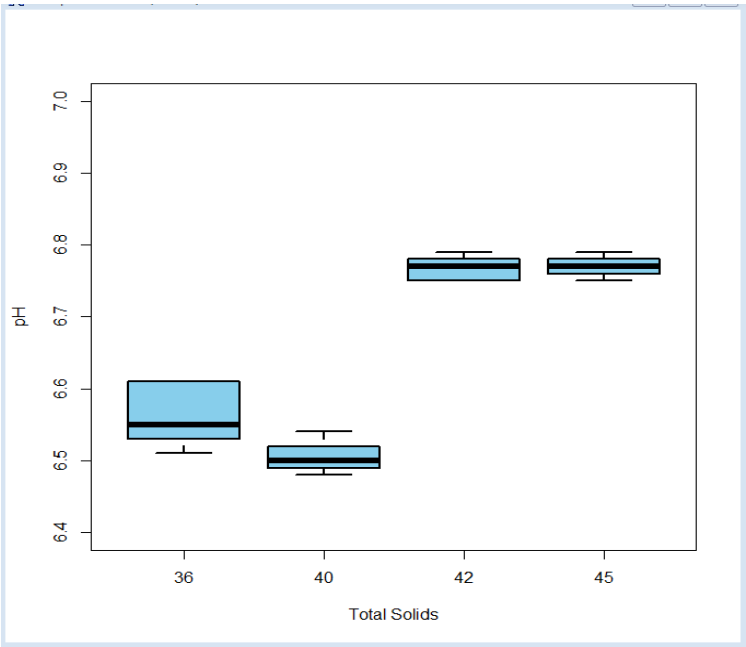
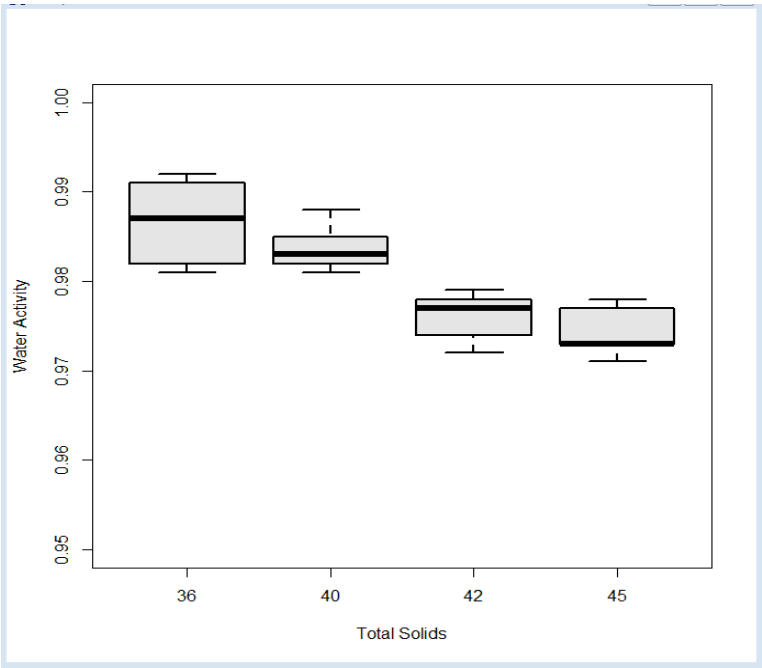


Figure 6B. Boxplot depicting interactions of total solids vs water activity**.**



CHAPTER 3

LISTERIA CROSS CONTAMINATION IN RAW ICE CREAM MIX CAN SERVE AS A PREDICTOR OF THEIR POTENTIAL PRESENCE AS HEAT- INJURED CELLS

NEHA

ABSTRACT

Listeriosis is a life-threatening infection caused by foods contaminated with *Listeria monocytogenes*. Some of the major ice cream recalls in recent years reaffirm the ability of this food borne pathogen to survive in diverse dairy processing environments and cause cross contamination. Inspection reports revealed lapses in implementing adequate hygienic practices for *Listeria* persistence in the processing environment, leading to cross contamination of ice cream. The higher levels of cross contamination of raw ice cream mix may result in some heat-injured cells, when exposed to minimum heat treatment (69°C for 30 min). These heat-injured cells could later recover under abused storage and handling conditions, and pose a health risk. Evidence about the presence of injured cells in ice cream mix may thus prove useful to establish the overall *Listeria* risk, which is the aim of this study. Challenge studies were conducted to evaluate the dose dependent presence of heat-injured cells of *Listeria*. Ice cream mix formulations of four different types (total solids% 36, 40, 42 and 45) were inoculated at 2.0, 3.0, and 4.0 log cfu/g levels of *Listeria innocua* (an established surrogate). The dose levels were selected based on a likely cross contamination on the raw side from environmental *Listeria*, especially due to their resident

nature and growth in harborage sites. The samples were exposed to minimum heat treatment (69°C for 30 min), and the survivors, including heat-injured cells, were enumerated using standard protocols. A binary logistic regression model was fitted for evaluating the severity of risk. The influence of total solids, water activity, and pH variability were also studied on *Listeria* survival. The enrichment protocol, using Buffered *Listeria* Enrichment Broth (BLEB), followed by plating on Modified Oxford Agar and Rapid *L' mono* medium, revealed the random presence of heat-injured cells in BLEB only at the highest dose level of 4+ logs. Any potential risk from heat-injured cells was thus limited only to the highest levels of cross contamination, irrespective of the type of the mix. Significantly, none of the pasteurized ice cream mix samples supported the recovery of any heat-injured cells of *Listeria* during 72h holding at 7°C, even at the highest dose level of 4+ logs, under the conditions of experimentation. The level of cross contamination (dose) emerged as a predictor of the potential presence of heat-injured cells of *Listeria,* exposed to minimum pasteurization treatment.

INTRODUCTION

Listeria monocytogenes is amongst one of the five most implicated food borne pathogens, known for its ubiquity, ability to withstand freezing temperatures and persistence in food processing facilities. The psychrotropic gram-positive pathogen grows between (-) 0.4 and 50°C, with optimum growth between 30–37°C, and at a neutral pH. It, however, can also grow well at a much lower pH range of 5.0 to 5.7 at 4°C, and 4.3 to 5.2 at 30°C (Farber and Peterkin,

1991*). Listeria* has become a greater challenge for the food industry as it can withstand different types of processing stresses, and can survive in moist environment, the conditions that commonly exist in dairy processing plants. The Food Advisory Committee of FDA has even established a zero-tolerance policy for *Listeria* in ready to eat products.

In the context of raw milk contamination, the prevalence of *L. monocytogenes* on cattle and small ruminant farms was reported to be 22.2 and 16.8%, respectively, in New York and adjacent states (Nightingale et al., 2004). Feeding cattle with poor quality silage, lack of following good practices during milking and insufficient cleaning of cows enhanced possibility of raw milk contamination with *L. monocytogenes* (Sanaa et al., 1993; Hunt et al., 2012). On the other hand, sometimes, mastitis, encephalitis and abortions due to *Listeria* infection in cattle can also results in shedding of organisms in the milk (Fleming et al., 1985), which in turn lead to the cross contamination of milk by *Listeria*. The intracellular presence of some *Listeria* spp., within leukocytes, might result in their greater ability to survive the minimum pasteurization temperatures (Doyle et al., 1987). For instance, in Massachusetts, an inadequate filtering system in a plant failed to clarify the milk prior to pasteurization, and introduced leukocytes into the system. This resulted in an inadequate pasteurization effect, hence, lead to an outbreak due to presence of intracellular lipid-shielded organisms (Bearns and Girard, 1958). With regard to ice cream, according to Centers for Disease Control (CDC), there were nine *Listeria* related ice cream recalls during 2014-2015. The Blue Bell incidence in March 2015 led to 10 hospitalizations and 3

deaths. Later in April 2015, JENI's splendid ice cream tested positive for *Listeria* and recalled all the products. During sampling of two dairy processing plants to evaluate the traditional and molecular methods for *Listeria* detection, it was observed that the molecular method depicted higher *Listeria* contamination within the processing environment. Some equipment within the dairy processing environment have previously been shown to be contaminated with *L. monocytogenes* to a level of 10^4- 10^5 cfu (Alessandria et al., 2010). In another recent recall involving Fieldbrook Foods ice cream bars, although no illnesses were confirmed, but the finished sample tested positive for *L. monocytogenes* (FDA 2018).

In the context of ice cream safety, pasteurization of ice cream mix is regarded as the terminal kill step. However, several studies also demonstrated that *L. monocytogenes* could survive a minimum HTST process; under certain conditions such as if relatively large numbers were present in milk, growth at elevated temperatures, level of background microflora, and certain ice cream constituents (Fleming et al., 1985; Doyle et al., 1987; Garayzabal et al., 1987; Bunning et al, 1988; Knabel et al., 1990; Farber et al. 1992; Holsinger et al. (1992)). In the study conducted by Doyle et al., *L monocytogenes* was isolated from milk in six of nine trials in which the heat treatment was 71.7° to 73.9°C for 16.4 s, with the *Listeria* population up to $4.8x10^4$ cells per mL of milk. Similarly, a study conducted by Farber et al., (1988) also revealed the significance of large inoculum size and potential survival abilities of the organisms during pasteurization. In addition to the inoculum size, the physiological state of the cells

and their heterogeneous response to stress also influence the survival of *Listeria* (Pascual et al., 2001). Holsinger et al. (1992), reported that while the guidelines for pasteurization time-temperature of ice cream mix adequately inactivated *L. monocytogenes*, but it is important to take every precaution to inactivate the organism, as some major ingredients of ice cream such as stabilizers are associated with their increased thermal tolerance, possibly through entrapment of the organism in the three dimensional network formed with casein micelle, as reported by Glicksman et al., (1983). While concluding the adequacy of pasteurization, Lou and Yousef (1999), also mentioned about the general use of pasteurization temperatures well above the minimum legal limit, being practiced by many raw milk processors. Additionally, Besse (2002) in his review mentioned that it was not feasible to eliminate all bacteria with thermal treatment, as heating lead to a physiological stress within cells leaving them injured. These injured cells could repair themselves once the favorable conditions are back and provide a threat to the product quality (McMahon et al., 2000). It is also important to mention that in most of these studies, either a general-purpose enumeration medium was used, or it was not clear if any selective enrichment protocol was adopted. Many previous studies have shown that enrichment process greatly influences the recovery of true numbers of *Listeria* (Lund et al., 1991; Warburton et al., 1991; Flanders eta al., 1994). Detection of any stressed and injured cells becomes further complicated due to diverse background microflora, and most of the general enumeration protocols may not prove to be as effective (Vlaemynck and Moermans, 1996; Pritchard and Donnelly, 1999). It would thus be useful to

evaluate any potential presence of heat-injured cells in ice cream mix exposed to minimum pasteurization treatment by specifically employing the standard enrichment protocol (FDA 2018), and not by simply relying on direct plating on general-purpose media. This approach can also help restrict the overgrowth of background microflora including *Bacillus* spp., which has been reported to inhibit the recovery and growth of heat-injured *L. monocytogenes* (Buchanan, 1990; Tharrington and Sorrells, 1992). Although, such heat-injured cells have so far not been reported to be the cause of any outbreaks, studying their presence can help us design more robust risk assessment protocols. In view of this, we aimed to study, if there is any possibility for detecting heat-injured cells of *Listeria* in the ice cream mix, when contaminated with large inoculum size (up to 4+ logs per gram of mix), subjected to minimum heat treatment of 69°C for 30 min, and enumerated by employing the FDA approved enrichment protocol. Emphasis was also given to study the chances of their recovery during later stages of handling and processing of pasteurized mix. Binary distribution models were developed by applying logistic regression, where the interactive effect of dose, ice cream mix storage temperature and storage duration, water activity, and pH was also evaluated on the presence and potential recovery of heat-injured cells.

MATERIALS AND METHODS

Types of Ice Cream Mixes and Basic Analysis

Source of Raw Ice Cream Mixes. Raw ice cream mix formulations of 36%, 40%, 42%, and 45% total solid (TS) levels were obtained in one-gallon packs, in temperature-controlled containers, from a commercial ice cream manufacturer.

The average fat content in these formulations were 7.2, 14.2, 16.3, and 16.4%, respectively. The average sugar content varied from 19.3% to 22.2%, while the protein content varied from 3.1 to 4.2%. The 45% TS mix samples contained 3% chocolate powder. These were analyzed, upon receiving, for background bacterial counts and chemical parameters such as water activity and pH. A 90 g portion of each ice cream mix sample was kept aside for conducting the *Listeria* challenge studies as detailed below. In all, three trials, each in the replicates of three, were conducted for each total solid level.

Microbiological Analysis of Ice Cream Mixes. To establish the background microflora, ice cream mix samples were plated on Brain Heart Infusion (BHI) agar (Oxoid, Thermo Scientific, UK) for aerobic plate counts by following standard methods (Wehr and Frank, 2004). Eleven grams of the respective sample was aseptically drawn and mixed with 99.0 mL of phosphate buffered saline (PBS), using a stomacher (Stomacher$^{@}$ 400 Circulator, Seward Laboratory System Inc., FL, USA) for 30 seconds to obtain the first dilution. Further serial dilutions were made, as necessary, in phosphate buffer saline (PBS), and plated on BHI Agar. The plates were incubated at 37°C and colonies were counted at 24-48h hours for obtaining aerobic plate counts.

Chemical Analysis of Ice Cream Mix Samples. The ice cream mix samples were tempered to ambient temperature. The water activity of the samples was measured using aqua lab CX-2 system (Aqua Lab, Decagon Devices, Inc.), and the pH was measured using A321 pH meter (Orion Star^{TM} A321, Thermo Scientific).

Challenge Studies using Listeria innocua

Sourcing L. innocua and Culture Propagation. Pure culture of *L. innocua* ATCC 33090, an established surrogate of *L. monocytogenes,* (Friedly et al., 2008 and Liu et al., 2009), was procured from American Type Culture Collection (Manassas, VA, USA). It was activated in BHI broth (Oxoid, Thermo Scientific, UK) by incubating at 37°C for 24 hours. In order to achieve the mid-exponential cells, the activated culture was sub-cultured in 9 mL of BHI broth, and the 6 h growth was pelleted out and suspended in PBS to get the desired numbers of cells for inoculation purposes. For long term storage, the pelleted culture was maintained in 1.8-mL cryogenic vials (Copan Diagnostic Inc., Murrieta, CA) that contained sterile beads and glycerol. The vials were stored in a NuAire ultralow deep freezer (NuAire Inc., Plymouth, MN) at −80°C until further use.

Inoculation and Pasteurization of Ice Cream Mix Samples. To study the dose response, ice cream mix samples were tempered to 40°C in a water bath (Wehr and Frank, 2004), and inoculated at the average dose levels of log 2.0, 3.0, and 4.0 cfu/grams of *L. innocua* activated culture as explained above. The 6 h grown culture was centrifuged (2000 x g for 15 min at 4°C) and washed twice with PBS to harvest the cells. Appropriate cell suspensions (2+, 3+ and 4+ logs cfu/grams) were made by diluting the harvested culture in PBS. The inoculation levels of raw ice cream mix samples were selected to simulate any potential environmental cross contamination situation, as discussed in the introduction section of this paper. The inoculated ice cream mix samples were minimally batch pasteurized at 69°C for 30 min (corresponds to 80°C for 25 seconds) (PMO, 2015) in a

shaker water bath (Lab companion AAH44063U 17L reciprocal shaking water bath, Cole- Parmer, USA). During heating, the core temperature was continuously monitored using the temperature probe. The samples were then rapidly cooled in an ice bath before further analysis. Each experimental trial was done in the replicates of three and repeated three times. The experiments were also repeated under the production environment by using HTST time temperature conditions to evaluate under production conditions.

Recovery of Survivors using Direct Plating Method. For each direct analysis, twenty-five grams of inoculated and pasteurized ice cream mix sample were drawn and suspended in 225 mL PBS, followed by further serial dilutions, as necessary. Appropriate dilutions were direct plated on an esculin-based medium, Modified Oxford Agar (MOX) to enumerate the *Listeria* cells that might have survived the heat treatment. The direct plating on Oxford agar likely supports the growth of uninjured populations (Hansen and Knochel, 2001). The plates were incubated at 37°C for 24 hours. The incubated plates were observed for typical *Listeria* colonies, which were small black colonies with a black halo (Park et al., 2013).

Recovery of Heat-injured Cells using the BAM Enrichment Protocol. Additionally, in order to enumerate any heat-injured cells of *Listeria,* an enrichment protocol was used as recommended by USFDA (Bacteriological Analytical Manual, BAM, 2018). A 25 grams portion each of the lab pasteurized inoculated sample was suspended in 225 mL of buffered *Listeria* Enrichment Broth (BLEB), and incubated for 4 hours at 30°C. *Listeria* selective enrichment

supplements (Acriflavin HCl, Nalidixic acid and Cycloheximide) were added after four hours and the samples were further incubated at 30°C. At 24 to 48 h intervals, BLEB enrichments were streaked on MOX agar and a chromogenic selective medium (RLM) to show the recovery of heat-injured cells. The typical *Listeria* colonies on MOX appeared black with black zones, and on RLM, the colonies were white, with or without halo. The results were recorded as positive or negative, based on the presence or absence of colonies on the selective media used for streaking, due to the recovery of heat-injured cells of *Listeria*.

Recovery of Heat-injured Cells in Ice Cream Mix itself. The inoculated and pasteurized ice cream mix samples were stored up to 72 h at 7°C (to simulate the common duration of ice cream mix storage in the ice cream industry, to observe the recovery of any heat-injured cells in the ice cream mix itself. In order to study this, the representative ice cream mix samples from each total solid level, in individual treatment bottles, were held at 7°C (Isotemp low temperature incubator, MDL 146E, Fisher Scientific) on a shaker. The samples drawn at the end of 72 h were serially diluted using phosphate buffer saline, followed by direct plating on MOX and RLM agar to enumerate any heat-injured *Listeria* cells that might have been recovered during the mix storage process in the ice cream mix samples itself.

MALDI-TOF based Identification of Isolates and Scanning Electron Microscopy (SEM) of Uninjured and Injured Cells. Selected isolates were streaked on RLM and MOX agar plates, and the pure colonies thus obtained were used for the confirmatory identification using matrix assisted laser

desorption ionization-time of flight (MALDI-TOF) (Thouvenot et al., 2018) (analysis conducted at the Diagnostic Lab, Veterinary and Biomedical Sciences Department, South Dakota State University).

In addition, the intact and injured *Listeria* cells were also observed under a scanning electron microscope (Hitachi S-3400N, Hitachi America Ltd., Tarrytown, NY), located in the Daktronics Engineering Hall, South Dakota State University. In order to study the morphological changes in heat injured *Listeria*, the bacterial suspensions in BHI broth were exposed to minimal thermal treatment, and the pre- and post-pasteurization suspensions were smeared on the glass slides. These smears were air-dried by following the air-drying method described by Hassan et al., (2010), with slight modifications. For SEM, the air-dried samples were sputter coated with a 10-nm-thick layer of deposition of 99% gold, and observed at 10 kV accelerating voltage by maintaining a distance of 10 mm from the slide.

Data Analysis and Developing Risk Assessment Models

Descriptive Analysis. Graphical descriptive analyses were performed to study the marginal and joint distributions of water activity and pH by total solids levels. Furthermore, we constructed a scatterplot of pH by water activity (grouped by total solids levels) to uncover if there was any pattern.

Statistical Analysis. The results related to plate counts were subjected to analysis of variance (ANOVA) using SAS® 9.3 (SAS Institute Cary, NC, USA). The level of significance was set to 5%.

Binary Logistic Regression Analysis. For the challenge studies, three dose levels (log 2.0, 3.0, and 4.0) were tested for thermal resistance and presence of any heat injured cells of *Listeria.* Ice cream mix samples with different levels of total solids (36, 40, 42 and 45%) were inoculated, and pasteurized at 69°C for 30 min, and direct plated on MOX agar for the presence or absence of survivors. In order to model the risk, the standard binary logistic regression was applied. The binary dependent variable included the absence of heat-injured cells, recorded as 0 (zero), and their presence (recovery) recorded as 1 (one). During further study using enrichment broth, the independent variables considered for the potential recovery of heat-injured cells were dose, temperature, water activity, and pH. At a later stage of the modeling process, penalized logistic regression was also used, due to the quasi-complete separation. Non-significant predictor variables were eliminated, and the water activity and pH data were used to study their interaction with different total solid levels to create a baseline.

RESULTS AND DISCUSSION

Influence of Ice cream Types on Microbiological Quality and Chemical Parameters of Ice Cream Mix Samples. Raw ice cream mixes of different types with total solids levels (36, 40, 42 and 45%) were evaluated for the initial bacterial loads and some chemical parameters. The average aerobic plate counts of raw ice cream mix samples varied within a narrow range from 4.65-5.67 $\log_{10}$ cfu/ gram. Our data related to aerobic counts in samples were observed to be consistent for all the three trials, conducted in the replicates of

three, for each formulation. This data helped us to establish a base value for the background bacterial load in raw ice cream mix samples tested. Such background counts in raw ice cream mix samples may result due to carry-over contamination from different ice cream mix ingredients, and the environmental cross contamination on the raw side of processing. Although we did not come across any other such reports on raw ice cream mix bacterial counts, even the processed ice cream has been shown to have range from 2- 5 logs (Campbell M., 2015). The raw ice cream mix, along with its natural background microflora was chosen for inoculation studies to simulate the commercial manufacturing conditions. The experiments were designed to potentially evaluate the effect of background microflora, if any, on the survival of heat-injured cells, when exposed to minimum pasteurization. This information would also be useful in future studies to evaluate their influence on the potential recovery of any heat injured cells during later holding of ice cream mix, under extended storage prior to further processing. The cross contamination of raw ice cream mix with *Listeria* and the later presence of heat-injured cells may also be influenced by the level of background microflora in the raw ice cream mix. With regard to the possibilities of *Listeria* contamination in raw ice cream mix, carryover of the pathogen in raw milk and potential cross contamination in the processing facility were hypothesized. In a previous study, about 18.8% of the equipment sites, 54.7% of the environmental sites, and 44.4% of the raw milk samples had *Listeria* in Northern Ireland (Fox et al., 2005). Similarly, another study done by O' Donnell in 1995 indicated that 15.43% of raw milk samples from bulk tanks in England and

Whales were contaminated with *Listeria spp.* The study also mentioned about a significant increase in *Listeria* population during a specific time of the year, when the cows were housed for that particular period. In addition, some of the previous reports depicted the presence of *Listeria* in the dairy processing environment, more prominently the wet drains and floors, and walls. It was thus inferred that the *Listeria* contamination is more likely to occur on the raw-side within an ice cream processing environment. Hence, in order to simulate the actual commercial conditions that might lead to *Listeria* cross contamination; raw ice cream mix samples were used along with background microflora, for this study. This approach also takes in to account any influence of background microflora, which might compete with *Listeria* contaminants within the ice cream mix matrix itself.

Chemical analysis of raw ice cream mix samples revealed that the water activity (a_w) varied within a narrow range from 0.97 to 0.98, and pH from 6.4 to 6.7 of the ice cream mix samples, as also reported by Gougouli et al., (2008. This helped us to establish that the ice cream mix samples selected for experimentation were within normal range of variations. The information on pH and water activity proved useful while evaluating the interaction of these two variables as covariates for developing regression models for the survival of heat-injured cells of *Listeria* due to the cross-contaminated ice cream mixes. The pH and a_w values were found in the typical range, as generally encountered in commercial manufacturing and showed least variability. The discussion in the

following sections further elaborates the interaction of these variables and their influence on any survival potential of heat-injured cells of *Listeria.*

Typical Colony Morphology of Listeria and Predominant Background Microflora. The *Listeria* selective, MOX and RLM agar, media proved very useful in isolating and differentiating *L. innocua* from the background microflora, which primarily included *Microbacterium paraoxydans* and thermoduric endospore formers of *Bacillus licheniformis*. A look at Figure 1 and Figure 2 reveals that typical colonies of *L. innocua* (the inoculated organism) appeared to be round, approximately 1.0 mm in diameter, black in color and surrounded by a black zone (Remel Manuals & Protocols), and white, with or without halo on RLM (Bio-Rad specification sheet). However, the typical colony morphology of the background species *Microbacterium paraoxydans,* appeared black with a black zone, approximately 4 mm diameter and was elevated on MOX, and the colonies were transparent and round on Rapid L' mono agar medium (the color of the medium turned pink) (Figures 3 and 4). Figure 5 depicts typical colony morphology of *Bacillus,* which appeared as scattered colonies with cottony appearance on MOX. Further confirmation of selected colonies was done using MALDI-TOF. This additional information can prove useful for further studies and prevent any false positive identification in the presence of background microflora.

In order to study any morphological changes in the heat-injured cells of *Listeria*, intact and injured *Listeria* cells were observed by gram stained smears, as well as, scanning electron microscope. It may be seen from Figures 6A, and 6B that the heat injured cells appeared to be shorter rods clumped together

having ruffled surfaces as compared to the intact (uninjured cells, pre-heat treatment). This observation was similar to a previous report, where the ultrastructure analysis of heat-injured cells indicated cytoplasmic clearance and swollen cell surfaces as an impact of heat injury, while there was no leakage of cellular components observed (Novak and Juneja, 2001). Such injured cells might recover on restoration of the adequate growth conditions such as temperature and composition of the product (Mackey et.al., 1994). Based on these important observations, our study emphasized the significance of the presence and recovery potential of heat-injured cells in order to establish their relationship with the cross-contamination levels (dose levels). This also helped us to generate evidence in support of the hypothesis that the presence of *Listeria* heat-injured cells is dependent on the levels of cross contamination, and such injured cells may serve as a determinant of risk from injured cells in ice cream mix exposed to minimum pasteurization.

Risk Analysis of Listeria Contaminated Raw Ice Cream Mix

The microbiological risk analysis models include elements such as hazard characterization and levels, potential exposure, and relative severity of risk (Codex, 1999; EPA, 2012). Keeping the fundamental risk analysis in mind, the overall objective of this study was to evaluate if the cross-contamination levels of *Listeria* in raw ice cream mix (representing any environmental cross contamination under the industrial processing) could influence their later survival as heat-injured cells (as shown by selective enrichment) against minimum pasteurization treatment.

Relationship of Cross-contamination Levels (dose response) with Intact (Uninjured) Cells. In the present study, the influence of dose level (representing potential environmental cross contamination situations) was evaluated on any intact cells. Results obtained by direct plating of thermally treated inoculated raw ice cream mix samples did not detect any survivors (uninjured intact cells) at any of the inoculation level of 2, 3 or 4 log cfu/ gram. Such enumeration protocols has previously been reported to be suitable for detecting uninjured populations (Hansen and Knochel, 2001). These observations also support some of the previous reports, which demonstrated that *L. monocytogenes* did not survive a proper pasteurization process in ice cream mix with $D_{79.4°C}$ value of 0.5 seconds (Bradshaw et al., 1987, Hosinger et al., 1992).

Relationship of Cross-contamination Levels (dose response) with Injured Cells. As the direct plating technique is usually not suitable for the recovery of stressed cells (Jasson et al., 2010), BLEB enrichment protocol is recommended (BAM, FDA) to estimate the presence of injured cells in a product matrix. Following the enrichment protocol, we detected a random presence of some heat-injured cells at the highest dose level of cross contamination (depicted by average log 4.0, inoculation levels) in all four types of ice cream mixes when subjected to minimum pasteurization treatment. As reported previously, this could be due to the sub lethal damage of cytoplasmic membrane, also known as structural damage (Hauben et al., 1996; Pagan and Mackey, 2000 and Ritz et al., 2001) or damage to intracellular components, also known as physiological damage (Niven et al., 1999 and Ritz et al., 2002). Since the heat-injured cells

were only detected at the highest inoculation levels, the average dose level of log 4.0 was considered for risk assessment and to evaluate its role as a predictor of the presence of heat-injured cells, when exposed to minimum pasteurization.

As *Listeria* is a high-risk pathogen, especially for pregnant women and elderly people (Choi et al., 2016), it is important to establish the accurate risk assessment models. Traditionally, a quantitative risk assessment model focuses on preventing, regulating and understanding the risk due to pathogenic microorganisms (EPA, 2012). However, such risk assessment models generally do not consider the presence and recovery potential of injured cells, and thus may not adequately address the risk. As per one such study (Roso, 1995) based on Monte Carlo simulations, 99% of the iterations showed less than 100 cells per serving (as low as 3.7 cells/ gram of *Listeria monocytogenes*). Our study thus provided significant information related to injured cells that could prove useful in conducting risk assessment for the process of ice cream mix storage and handling. Such heat-injured cells may have the potential to recover and cause a risk (Bunduki et al., 1995). The dose (depicting the level of potential cross contamination from the processing environment on the raw side) emerged as a predictor of the random presence of heat-injured cells. A previous report by Chen et al., (2017) for creating a probabilistic model and linking it to the bacterial numbers, also depicted the importance of using selective medium to recover low number of uninjured cells of *Listeria*. Hence, our study puts forth a case for emphasizing the significance of using enrichment protocol to enumerate any

random presence of heat-injured cells in developing more robust risk assessment approach.

Recovery of Heat-Injured Cells in Ice Cream Mix itself. At this point in the study, it became clear that only high level of cross contamination (log 4.0 or higher) could result in post-pasteurization detection of some heat-injured cells from the inoculated ice cream mix, when subjected to minimum pasteurization. However, the detection of heat-injured cells was only accomplished by using the selective enrichment protocol using BLEB (BAM protocol) under the optimal growth conditions of organism. In order to create a more realistic assessment for the ice cream industry, the recovery potential of heat-injured cells of *L. innocua* was evaluated within ice cream mix itself under the mix aging and storage conditions. The inoculated and pasteurized ice cream mix samples of different types, with total solid levels (36%, 40%, 42%, and 45%) were stored at 7°C up to a maximum duration of 72 h (PMO, 2015). Significantly, none of the samples showed recovery of any heat-injured cell in the ice cream matrix itself, during the entire process of mix storage, under the conditions of experimentation. It thus reassures the safety of the standard ice cream manufacturing process. However, further studies in this direction are necessary to clearly understand the role of the random presence of heat-injured cells in developing risk analysis to prevent any unintended consequences, especially during mishandling and any abused conditions.

Influence of Covariates on Risk Assessment. The interactions of all the covariates (dose, TS, temperature and duration of mix storage, water activity,

and pH), and their influence on the recovery of heat-injured cells was also studied by fitting a binary logistic model. In order to establish any relationship of pH and water activity with total solids, their interactions were studied. Evidently, pH differentiated itself with the total solids, as the higher total solid had slightly higher pH and the lower total solid formulations had a pH value on the lower side. While, as expected, with the increasing total solid levels, there was a decreasing trend in the water activity. These parameters were further studied for developing the interaction of the covariates using a mix plot, and two different clusters, based on the pH and $a_{w,}$ were observed (Figure 7). However, as the values within these two clusters were very close, they did not play any important role in predicting the risk from *Listeria.* Hence, despite having slight variability in pH and a_w, and their interaction with total solid levels, they did not appear to influence the overall occurrence of heat-injured cells, which was observed to be equal for all formulations. A previous report on ice cream mix components also concluded that the ice cream mix components showed no predictable influence on thermal stability of *L. innocua* (McKellar et al., 1996). These baselines values would be helpful in creating comparisons with temperature abuse studies, related to the interaction of covariates on the recovery potential of any heat-injured cells.

Industrial Application of Considering the Presence of Heat-Injured in Risk Analysis. The findings from our study can serve as a novel approach for *Listeria* risk analysis in the ice cream industry by considering the potential of random presence of heat-injured cells, using enrichment protocols. As both intrinsic as well as extrinsic factors, related to ice cream manufacture are critical to

comprehensively evaluate the risk from *Listeria,* and to develop efficient risk assessment models, it would be justified to incorporate heat-injured cells and their recovery potential in developing these models. To elaborate this point further, as the ice cream manufacturing involves several processing stages such as receiving raw ingredients, pasteurization, mix storage, freezing, hardening, and retailing, therefore, it is necessary to predict the fate of any cross-contamination of *Listeria* at each processing stage. The present study showed the random presence of heat-injured cells, through enrichment protocols, only at the high level of cross contamination (logs 4 or higher), when exposed to minimum pasteurization treatment. Under the conditions of experimentation, such heat-injured cells did not show any recovery during the normal storage of ice cream mix. However, the potential of injured cells to recover under any abused conditions cannot be ignored and needs to be investigated further.

CONCLUSIONS

Many ice cream related outbreaks have occurred in the recent past, mostly due to cross contamination under unhygienic processing conditions. *Listeria* is a very resilient organism, which can withstand adverse conditions such as wide ranges of pH, water activity, and low temperatures. Its persistence in the dairy processing environment has been reported, as the organism can form resistant biofilms leading to potential cross contamination of the product, especially from harborage sites. Its ability to withstand adverse processing conditions may also lead to stressed and injured cells of *Listeria,* which may

recover under abused conditions of handling and storage. Although such injured cells have not been linked to any disease outbreaks so far, this information could be helpful in improving risk assessment protocols. Keeping this in mind, the current study was conducted, which focused on the relationship of cross contamination levels at raw ice cream mix stage, with that of the presence and recovery potential of heat-injured cells using enrichment protocols. When the raw ice cream mix samples were inoculated with about log 4.0 cfu / gram of *Listeria innocua* (an established surrogate) and heat-treated to minimum pasteurization treatment, the direct plating did not detect any survivors. On the other hand, BLEB enrichment protocol detected a random presence of heat-injured cells. Statistical algorithms were used to fit a model by considering covariates. Based on the quasi-complete separation, dose (level of cross contamination) came up as a predictor of the presence of heat-injured cells, only at the higher level of cross contamination (average log 4.0 or more). Further studies are necessary to evaluate the significance of the possibilities of random presence of heat-injured cells in ice cream mix, when subjected to minimum pasteurization treatment. Importantly, ice cream mix itself did not support the recovery of any heat-injured cells, during common aging and storage conditions (7°C for 72h), while the influence of any abused handling conditions is yet to be studied.

Chapter 3 Figures.

Figure 1 and 2. Typical colonies of *Listeria innocua* (the inoculated organism) appeared to be round, approximately 1.0mm in diameter, black in color and surrounded by a black zone on Modified Oxford agar (MOX) medium and round, white, with or without halo on Rapid L'mono (RLM) agar medium.

1

2

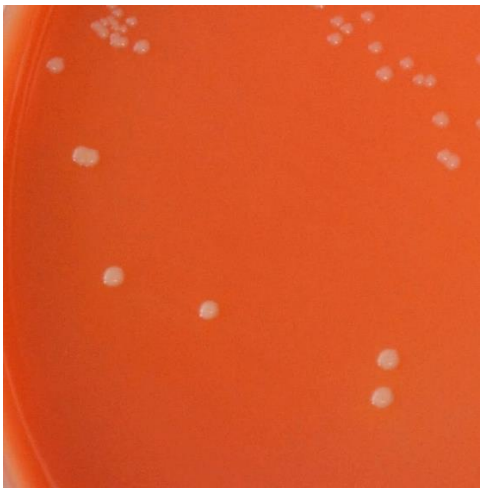
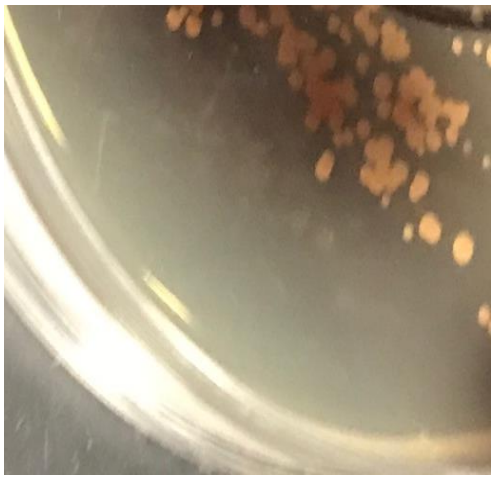


Figure 3 and 4. Typical colony morphology of *Microbacterium,* which appeared black with a black zone, approximately 4 mm diameter, and elevated on Modified Oxford agar medium. These colonies appeared transparent and round on Rapid L' mono agar medium (the color of the medium turned pink).

3



4

Figure 5. Typical colony morphology of a contaminant, *Bacillus* sp., which appeared as scattered colonies with cottony appearance on MOX.

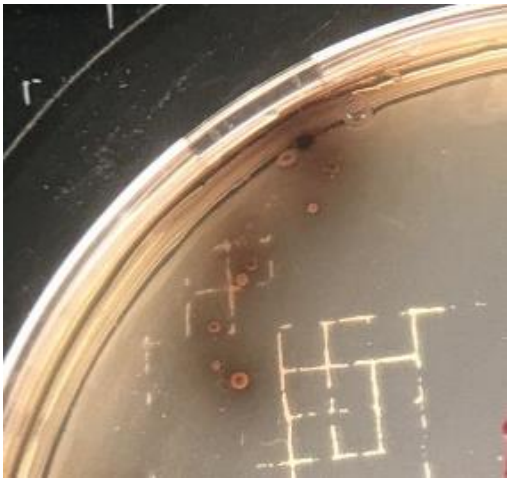
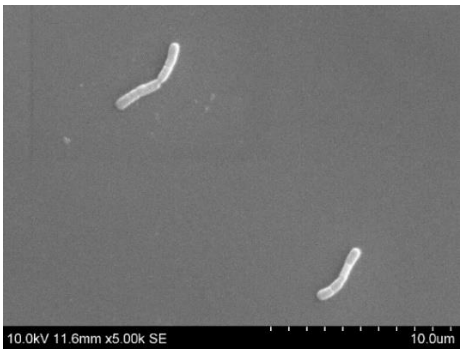


Figure 6. Scanning Electron Microscopic images of *Listeria innocua in BHI broth.* (Magnification: 10K); Intact cells SEM image (A), and Thermally-treated cells (B).

A



B

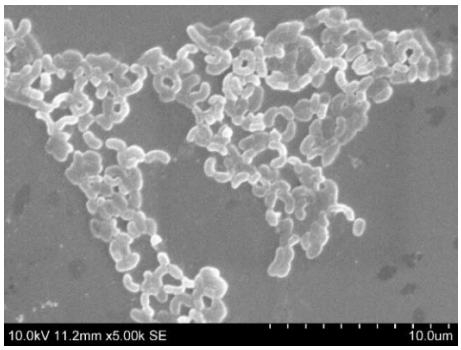
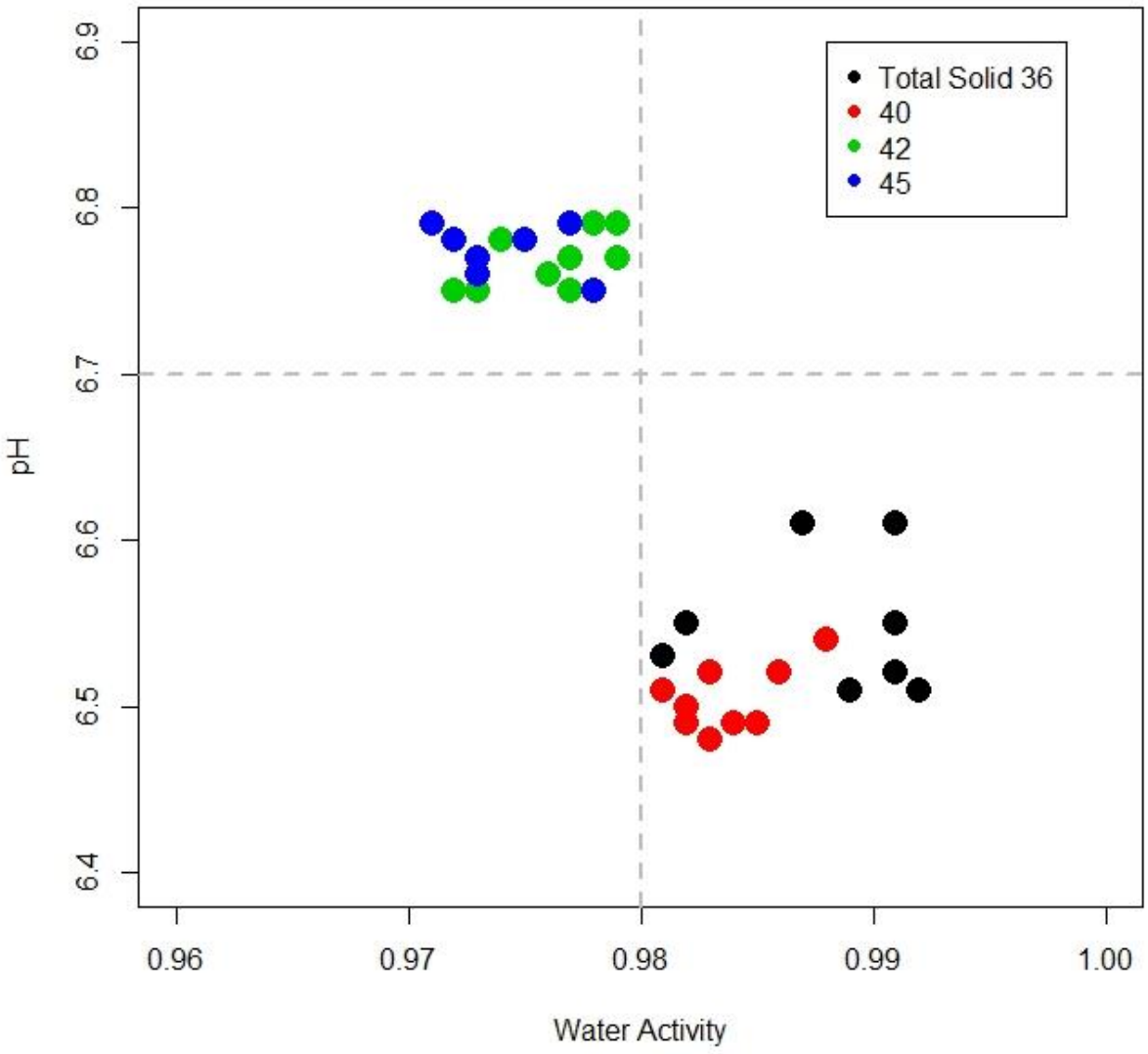


Figure 7. Scatterplot depicting clusters based on total solid levels.



CHAPTER 4

ENTRAPMENT OF *LISTERIA* CELLS WITHIN AIR CELLS OF ICE CREAM MIX MATRIX MAY LEAD TO HEAT-INJURED CELLS

NEHA

ABSTRACT

In our previous study, a random presence of heat-injured cells of *Listeria innocua* was observed in the ice cream mix, subjected to minimal heat treatment. Such heat-injured cells were recovered only by the selective enrichment protocol, while direct plating did not detect any *Listeria* cells. Although, such injured cells have so far not been reported to cause any disease outbreaks, it is important to understand their survival in food matrices such as ice cream that has recently been involved in some recalls. Through the present study, we propose a novel concept involving the possible protective role of air cells in the random presence of heat-injured cells. Challenge studies were conducted by inoculating ice cream mix samples (42% total solids, 16.3% fat, 22.2% total sugar, and 3.4% protein levels) with *Listeria innocua* (an established surrogate) at a mean spiking level of log 4.0 cfu/g. The inoculated samples were subjected to minimum batch pasteurization treatment at 69°C for 30 min, and any heat-injured cells were enumerated using buffered *Listeria* enrichment broth (BLEB), followed by plating on Modified Oxford (MOX) and Rapid'LMono (RLM) agars. Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM) were conducted on the air-dried, spiked ice cream mix samples, at pre- and post- thermal treatment stages. A random presence of heat-inured cells was confirmed by the BLEB

enrichment protocol, while direct plating did not reveal the presence of any intact cell survivors in heat-treated ice cream mix samples. The scanning electron microscopy (SEM) showed the presence of air cells in the ice cream mix samples. The pre-heat-treated spiked mix samples showed *Listeria* cells that were randomly distributed in the mix matrix, and also entrapped within some of the larger air cells. On the other hand, the post-heat-treated mix SEM and AFM micrograph showed entrapped cells only within the larger air cells. The mix matrix, on the other hand, did not show the presence of any *Listeria* cells. These observations thus suggest that the *Listeria* cells entrapped within the larger air cells might not have received adequate thermal effect and resulted in their random presence as heat-injured cells, as detected by enrichment protocol.

INTRODUCTION

Listeria monocytogenes is an important food borne pathogen that is frequently associated with recall of frozen products, and in recent years, even ice cream has been implicated. In the case of ice cream, according to Centers for Disease Control and Prevention (CDC), there were nine *Listeria* related ice cream recalls during 2014-2015. An incidence involving ice cream related outbreak in March 2015 led to 10 hospitalizations and 3 death. Later in April 2015, another commercial ice cream tested positive for *Listeria* and recalled all the products. More recently in 2017, a voluntary recall of all orange cream bars and chocolate-coated vanilla ice cream bars was reported, due to the possibility that they may be contaminated with *Listeria monocytogenes*. Post processing

environmental contamination was the likely cause of such incidences. On the other hand, the possibilities exist for a sublethal thermal effect, especially during batch pasteurization in small processing set ups, leading to injured cells. The intracellular packaging of some *Listeria* spp., within leukocytes, has been reported to result in an inadequate pasteurization effect, hence, leading to presence of intracellular lipid-shielded cells (Bearns and Girard, 1958). Similarly, significance of large inoculum size in potential survival abilities of the organisms during pasteurization have also been reported (Farber et al., (1988). Previous researchers have also reported that it was not feasible to eliminate all bacteria with thermal treatment (Besse 2002). The physiological stress caused by heating might lead to injured cells that could repair themselves when subjected to favorable conditions (McMahon et al., 2000, Besse 2002). In a study by Doyle et al., (1987), it was concluded that the *L. monocytogenes,* residing within polymorphonuclear leukocyte in the milk of *Listeria* - infected cows, could withstand minimum thermal treatment of 72.2°C for 16.4 seconds in a HTST pasteurizer. While concluding the adequacy of pasteurization, Lou and Yousef (1999) mentioned that ‘pasteurization is a safe process, which reduces the number of *L monocytogenes* occurring in raw milk to levels that do not pose an appreciable risk to human health’. They also noted that ‘although minimum HTST milk pasteurization is considered a safe process, most raw milk processing facilities have wisely adopted pasteurization temperatures well above the minimum legal limit’. This necessitates studies to evaluate processing factors that may have the potential to result in subthermal effect leading to injured cells. In a

previous study in our lab (Neha et al., 2018), we observed the random presence of heat-injured cells of *Listeria* in ice cream mix, subjected to a batch heat treatment (69°C for 30 min). Such heat-injured cells were recovered only by the selective enrichment protocol, while direct plating did not detect any *Listeria* cells. Although, such injured cells have so far not been reported to cause any disease outbreaks, it is important to understand their survival strategies. Through the present study, we propose a novel concept involving a possible protective role of air cells resulting in the random presence of heat-injured cells.

MATERIAL AND METHODS

Challenge Studies

Raw ice cream mix samples (42% total solids, 16.3% fat, 22.2% total sugar, and 3.4% protein levels) were obtained in 3.76 Liter packs, in temperature-controlled containers, from a commercial ice cream manufacturer. These were analyzed, upon receiving, for background bacterial counts and chemical parameters such as water activity and pH using standard methods as explained in our previous paper (Neha et al., 2018). Pure culture of *Listeria innocua* ATCC 33090 was procured from American Type Culture Collection (Manassas, VA, USA). This is a nonpathogenic thermal processing surrogate of *L. monocytogenes,* which has previously been used in thermal resistance studies (Ryser and Marth, 1999, Kozempel et al., 2000, Li et al., 2013). It was activated (Neha et al., 2018) in brain heart infusion (BHI) broth (Oxoid, Thermo Scientific, UK) by incubating at 37°C for 24 h. In order to achieve the mid-exponential cells,

the activated culture was sub-cultured in 9 mL of BHI broth, and the 6 h growth at 37°C was pelleted out and suspended in PBS to get the desired numbers of cells for inoculation purposes. For challenge studies, ice cream mix samples were tempered to 40°C in a water bath (Wher and Frank, 2004), and inoculated at the average dose levels of log 4.0 cfu/g of *L. innocua* activated culture as explained above. The 6 h grown culture was centrifuged (2,000 x *g* for 15 min at 4°C) and washed twice with phosphate buffered saline (PBS) to harvest the cells. Appropriate cell suspensions were made by diluting the harvested culture in PBS to obtain log 4.0 cfu/g spiking levels in ice cream mix samples as per previous study (Neha et al., 2018). The spiked ice cream mix samples were heat treated at 69°C for 30 min in a shaker water bath (Lab companion, reciprocal shaking water bath, Cole-Parmer, USA) to simulate batch pasteurization in small processing facilities. During heating, the core temperature was continuously monitored using a temperature probe. The samples were then rapidly cooled in an ice bath before further analysis. Each experimental trial was done in the replicates of three and repeated three times.

Enumeration of Listeria

For enumerating intact cells, twenty-five g of spiked and pasteurized ice cream mix sample were drawn and suspended in 225 mL PBS, followed by further serial dilutions, as necessary. In addition, one mL each of spiked and pasteurized ice cream mix samples were direct plated on an esculin-based medium, Modified Oxford Agar (MOX), containing 1% Moxlactam selective supplement, to detect the *Listeria* cells that might have survived the heat

treatment. The direct plating approach helped to detect, as low as, one cell per g. The plates were incubated at 37°C for 24 h, and extended incubation of 48 h was used to confirm the absence of growth. The incubated plates were observed for typical *Listeria* colonies, which were small black colonies with a black halo (Park et al., 2013). To enumerate any heat-injured cells of *Listeria,* an enrichment protocol was used as recommended Bacteriological Analytical Manual, USFDA (Hitchins et al., 2018) with slight modification. A 25 g portion each of the lab pasteurized spiked sample was suspended in 225 mL of buffered *Listeria* Enrichment Broth (BLEB), and incubated for 4 h at 30°C. *Listeria* selective enrichment supplements (Acriflavin HCl, Nalidixic acid and Cycloheximide) were added after 4 h and the samples were further incubated at 30°C. At 24 to 48 h intervals, BLEB enrichments were streaked on MOX agar and a chromogenic selective medium (RLM) to show the recovery of heat-injured cells. The results were recorded as positive or negative, based on the presence or absence of colonies on the selective media used for streaking, due to the recovery of heat-injured cells of *Listeria*.

Microscopic Observations

To observe the air cell and any entrapped *Listeria* cells within, the pre- and post-pasteurized spiked ice cream mix suspensions were placed on the glass slides and air-dried by following a method previously described by Hassan et al., (2010) for membrane biofilms, with a slight modification. The air-drying process helped to retain the original distribution pattern of the spiked organisms in the mix matrix to the most extent. For SEM imaging, the air-dried samples

were sputter coated with a 10-nm-thick layer of deposition of 99% gold, and observed at 10 kV accelerating voltage by maintaining a distance of 10 mm from the slide (Hitachi S-3400N, Hitachi America Ltd., Tarrytown, NY). For the atomic force microscopy (AFM) studies, no pre-treatment was required for the air-dried samples. All the observations were made using a commercial Agilent 5500 scanning probe microscope using a method previously standardized in our lab (Marka and Anand, 2018), and Electrical Engineering department (Fan et al., 2018). The imaging was done in a tapping mode using Si cantilevers (Budget Sensors TAP 300 Al) AFM tip. The air-dried mix sample on the glass slide was mounted on the sample stage by sticking four corners of the membrane with scotch tape on the stage. The scanner was positioned at the flat center of sample surface. The tip's first resonance (f1) frequency of 67 kHz was fed into the first lock-in amplifier (LIA1). The vertical tip-sample separation was controlled from LIA1, which provided the error in the amplitude signal at f1 to the servo. This first lock-in amplifier was used for topographic and phase imaging. The tip characteristics used for measurement is Multi75E-G budget sensor with Cr/Pt coating and resonance frequency 75 kHz and force constant 3N/m. All images were stored in ASCII format and Gwyddion software (http://gwyddion.net/) was used to process the images in 2D format. A minimum of three scans were performed on each sample to observe the entrapped cells in air cell. Three trials in all were conducted for each of the experiment.

RESULTS AND DISCUSSION

Bacterial counts and chemical parameters obtained in the ice cream mix samples were quite comparable to our previous study (Neha et al., 2018). Results obtained by direct plating of heat-treated spiked ice cream mix samples did not detect any survivors (uninjured intact cells). These observations also support our previous report (Neha et al., 2018), and some others, which demonstrated that *Listeria monocytogenes* did not survive a proper pasteurization process in ice cream mix with $D_{79.4°C}$ value of 0.5 seconds (Bradshaw et al., 1987; Holsinger et al., 1992). Following the BAM enrichment protocol, we detected a random presence of some heat-injured cells. As reported previously, this could be due to the sub lethal damage of cytoplasmic membrane, also known as structural damage (Hauben et al., 1996; Pagan and Mackey, 2000 and Ritz et al., 2001) or damage to intracellular components, also known as physiological damage (Niven et al., 1999 and Ritz et al., 2002).

Role of Air Cell Entrapment as a Possible Protective Effect

The SEM observations helped us to establish the presence of several air cell in the ice cream mix samples. It was interesting to see that, in general, while most of the air cell were smaller (1-2 µm (Figure 1), some were larger (4-8 µm) (Figure 2). Such variability in the size of air cell could have been created during the handling stages of mix. It appears that the size of the air cell influenced the extent of entrapment of *Listeria* cells, which explains the random presence of heat-injured cells in the spiked heat-treated mix. Figure 3 and 4 show the presence of *Listeria* cells embedded in the matrix of spiked ice cream mix, as

well as, entrapped within the larger air cells, which are fused with the matrix. However, it may be seen that the smaller air cell was empty. This helped us prove the hypothesis that it was possible for the larger air cells within the ice cream mix to entrap the intact *Listeria* cells, in case any cross contamination of mix occurred prior to pasteurization. We further propose that any air cell-entrapped organisms might not experience the complete thermal effect, which needs to be further investigated. This would thus lead to their presence in the heat-treated mix in the form of heat-injured cells. In addition, due to the presence of some oxygen in the air cell, there may be conditions available for them to survive, while they may not multiply or repair. This observation is supported by a previous finding by Fanelli and McKee (2008), who reported living bacteria in ice cores sampled at depth of 4 kilometers in Antarctica. Such survival was indicated to be supported by oxygen, hydrogen, methane and many other gases that diffused from the air bubbles to the tiny liquid film that formed around microbes. It was interesting to note that virtually any microbe can remain alive in solid ice, resisting temperatures down to -55° C and pressures of 300 atmospheres (Fanelli and McKee, 2008).

The scanning electron and atomic force micrograph developed on the spiked and heat-treated ice cream mix samples helped us to prove this point further. We could observe some heat-injured *Listeria* cells entrapped within the larger air cell (Figure 5 and 6), while no such cells were visible in the surrounding matrix of spiked heat-treated mix. These SEM and AFM visual observations supported the culture-based data, as direct plating did not reveal any intact

Listeria cells in the spiked and heat-treated ice cream mix, while the enrichment protocol revealed the random presence of heat-injured cells. These observations thus suggest that the *Listeria* cells entrapped within the larger air cell might not have received complete thermal effect, thus resulting in their random presence as heat-injured cells.

CONCLUSIONS

Finding from our study suggest a possible role of air-pocket entrapment in protecting *Listeria* cells by limiting their exposure to the complete thermal effect. This information thus provides another perspective around *Listeria* risk, in addition to the other reported factors such as higher cross contamination levels, background microflora, product constituents, lipid-shielding effects, and entrapment in polymorphonucleocytes, reviewed in our previous paper (Neha et al., 2018). We hope that this additional information about air cell entrapment may serve to develop more robust *Listeria* risk-assessment protocols by considering the potential presence of heat-injured cells. This is an early evidence proposing the implications of air cells entrapped *Listeria* on their later detection as heat-injured cells in the heat-treated viscous food matrices such as ice cream mix. Further studies are necessary to evaluate the complete extent of these findings and their relationship to any injured cells detection.

Chapter 4 Figures.

Figure 1. SEM image showing small air pockets in the ice cream mix.

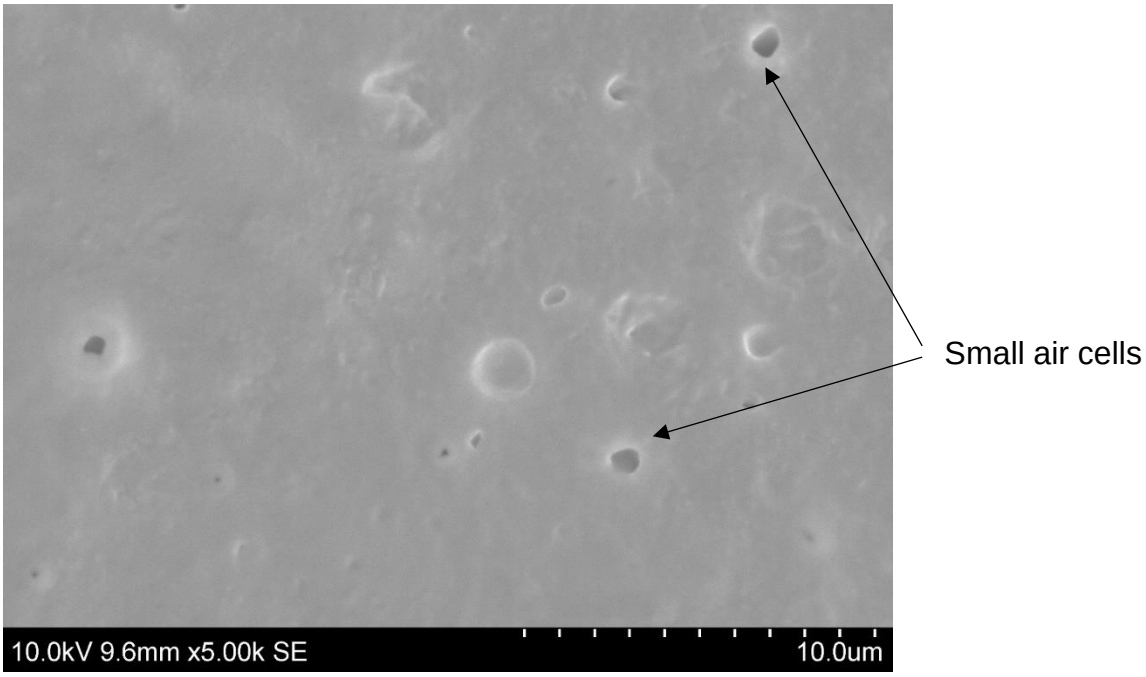


Figure 2. SEM image showing large air pockets in the ice cream mix.

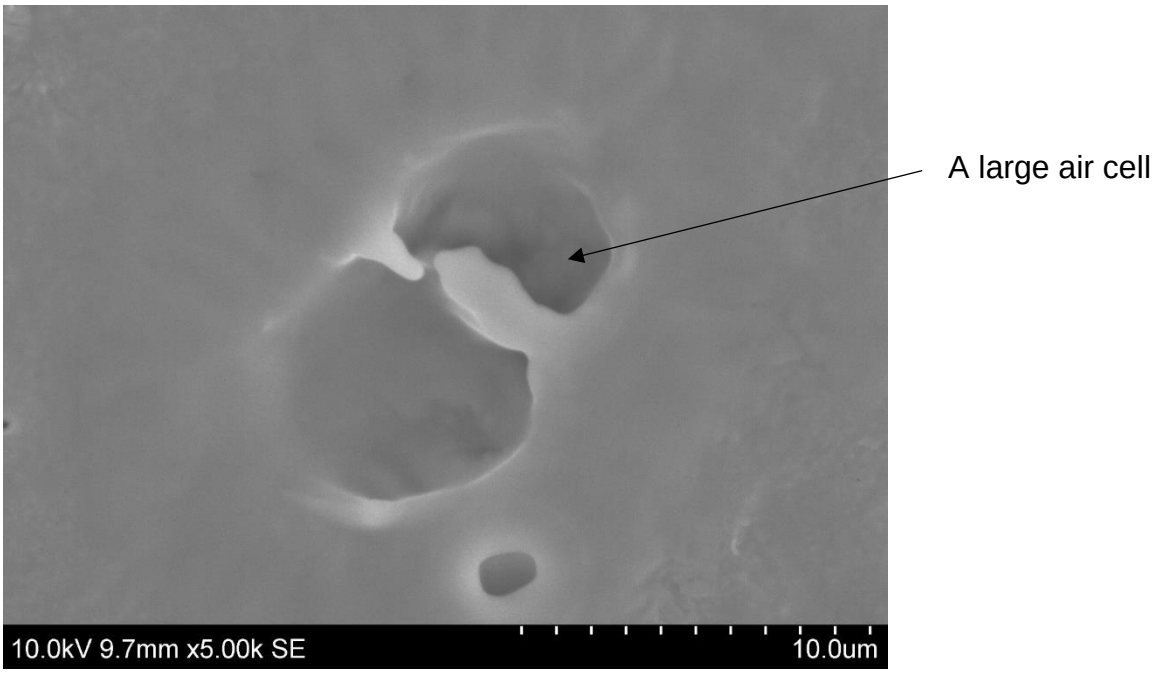


Figure 3. *Listeria* cells embedded in the matrix of spiked ice cream mix prior to heat treatment (SEM image).

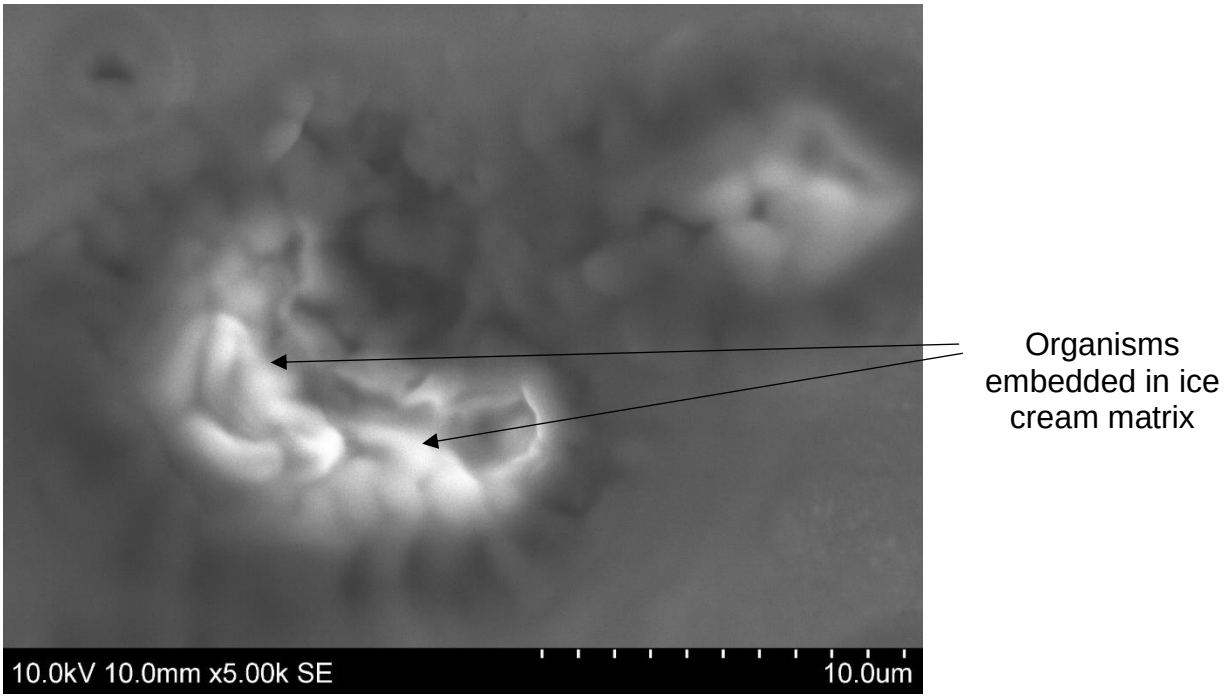


Figure 4. *Listeria* cells embedded in the matrix of spiked ice cream mix prior to heat treatment (SEM image) (Note the cells entrapped in large air cell fused with matrix, while small air cells are empty).

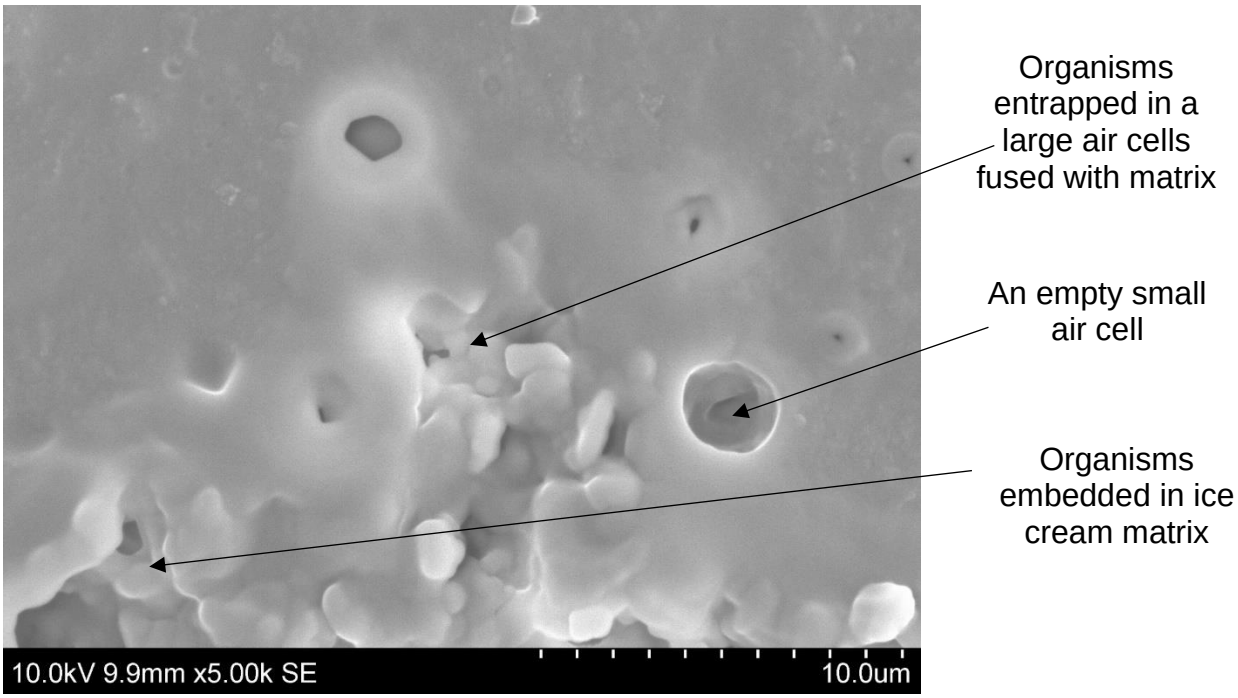


Figure 5. *Listeria* cells (heat-injured) entrapped in the large air-pocket of spiked ice cream mix after the heat treatment (SEM image) (no cells embedded in the matrix).

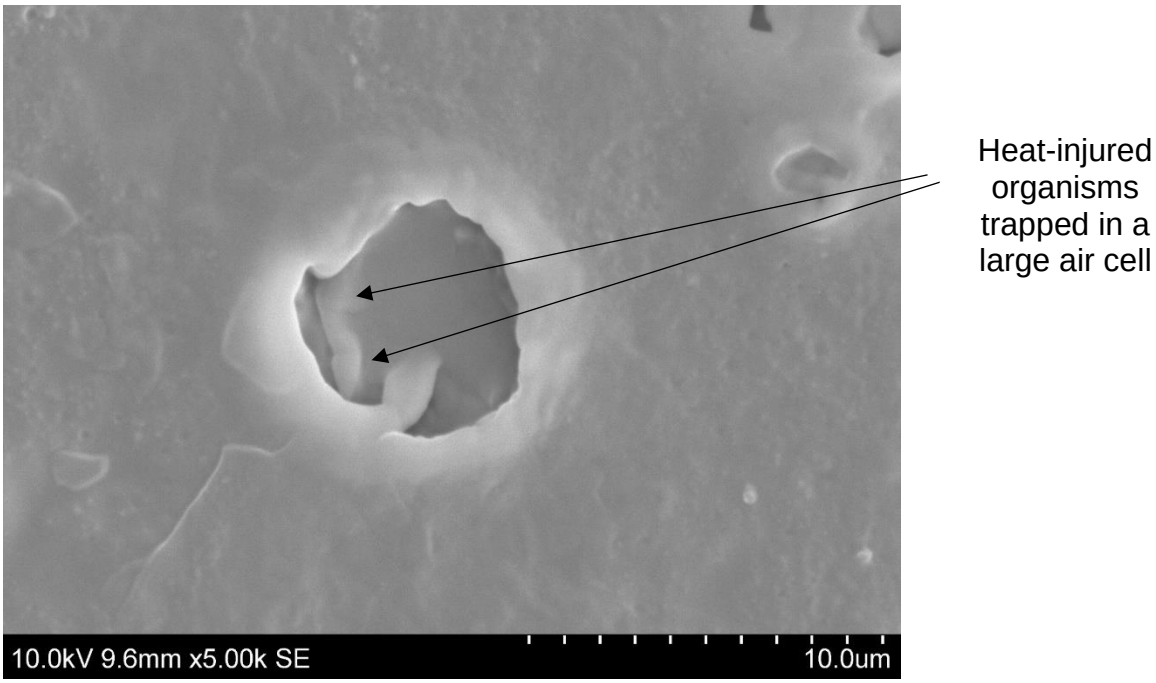
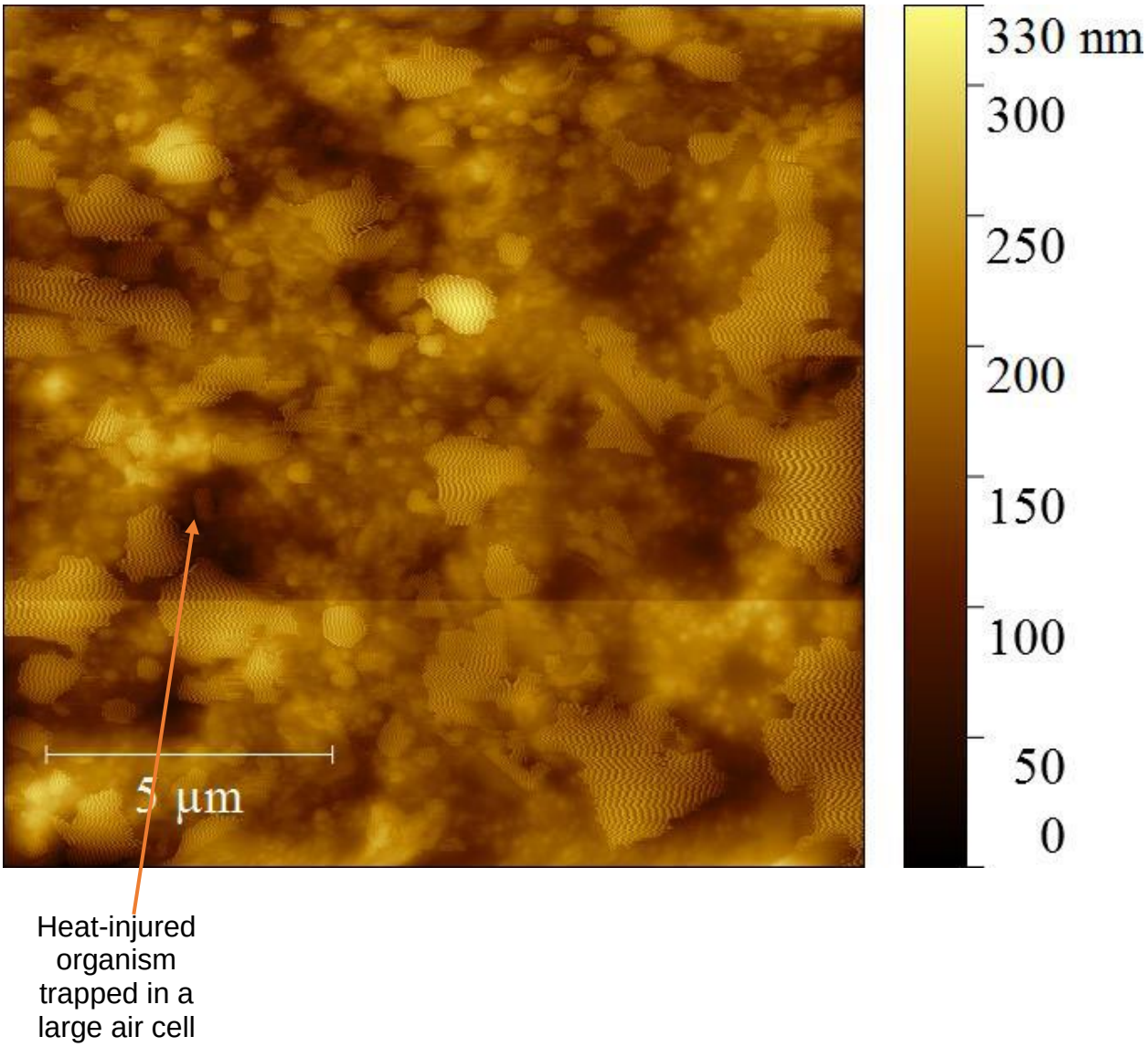


Figure 6. *Listeria* cells (heat-injured) entrapped in the large air-pocket of spiked ice cream mix after the heat treatment (AFM 2D image, color version available online) (no cells embedded in the matrix).



CHAPTER 5

RECOVERY POTENTIAL OF HEAT-INJURED CELLS OF *LISTERIA* UNDER PRODUCT ABUSE CONDITIONS VS. SIMULATED GASTRO-INTESTIONAL FLUIDS- A PROOF OF CONCEPT STUDY

NEHA

ABSTRACT

Product manufacturing relies on pasteurization to inactivate any food pathogens. However, factors such as cross contamination levels, and protection in product matrices may lead to sub lethal thermal effects, which may lead to cellular injury. One of our previous studies demonstrated the random presence of injured cells of *Listeria* in spiked ice cream mix samples, exposed to minimum pasteurization. Such injured cells did not show any recovery within the ice cream mix itself under normal handling conditions. The present study compares the effect of any temperature abuse of ice cream with exposure to simulated gastro-intestinal fluids on the recovery potential of any carried over injured cells. In order to generate heat-injured cells, raw ice cream mix (42% TS) samples were spiked with 4.54±0.13 log per gram of *Listeria innocua* (a surrogate) and subjected to pasteurization (69°C for 30min). For the intact cells study, 2.65±0.07 log per gram of *L. innocua* were spiked in the pasteurized mix. The mixes containing injured and intact cells were followed through ageing (72h at 7°C), freezing (-3.3°C), and hardening (- 40°C/ 12 h) steps. Direct plating, on *Listeria* selective agars was used for enumerating intact cells, while the heat-injured cells were recovered using *Listeria* enrichment broth (BLEB) prior to enumerating on

selective media. All trials were conducted in triplicates and data were statistically analyzed. Although no intact cells were observed, the enrichment protocol revealed the random presence of heat-injured cells at the post-pasteurization stages of processing. Freezing and hardening steps did not appear to have any further detrimental impact on heat-injured cells, carried over from the pasteurized ice cream mix. The temperature abuse conditions, evaluated in the current study, although led to pudding consistency, did not support the recovery of heat-injured cells. This implies that post-pasteurization contamination with intact *Listeria* might pose a greater risk than any carried over injured cells. Similarly, such injured cells did not show any recovery in the simulated gastro-intestinal fluids tested under in vitro conditions. In case of spiked intact cells, no detrimental effect of freezing and hardening steps was observed. This implies that post-pasteurization contamination of mix might pose a greater risk. Results from this study emphasize a need to design stage-specific critical control points to prevent any potential *Listeria* outbreaks.

INTRODUCTION

A common method used to inactivate bacterial contaminants in food products is the application of thermal treatments such as pasteurization. However, it is not always feasible to completely eliminate all bacterial contaminants with thermal treatments, especially at their minimum thresholds, as bacteria may experience physiological stress leading to cellular injury (Besse, 2002). Such injured cells offer a food safety risk by repairing themselves, once

the favorable conditions are restored (Bunduki et al., 1995; McMahon et al., 2000). As high-risk food pathogens such as *Listeria*, provide enhanced risk especially to pregnant women and elderly people (Choi et al., 2016), it is important to establish the accurate risk assessment protocols. Traditionally, a quantitative risk assessment model focuses on preventing, regulating and understanding risk due to pathogenic microorganisms (EPA, 2012). However, such risk assessment models generally do not consider the presence and recovery potential of injured cells, and thus may not adequately address the risk. Although, recovery potential of such injured cells or their association with any outbreaks has so far not been reported, in a study based on Monte Carlo simulations ((Roso, 1995), 99% of the iterations showed the significance of less than 100 cells per serving (as low as 3.7 cells/ gram) of *Listeria monocytogenes*. On similar lines, in our previous study, we reported the potential of randomly observing heat-injured cells of *Listeria innocua* in spiked ice cream mix that was exposed to minimum pasteurization treatment (Neha et al., 2018). The presence of injured cells was limited to the highest levels of inoculation (logs 4 cfu/g or higher), and none of the pasteurized ice cream mix samples supported the recovery of any heat-injured cells of *Listeria* during later holding of mix at 7°C for 72h. However, the potential of injured cells to recover under any abused ice cream handling conditions cannot be ignored, and hence this aspect was investigated in the current study. Another important aspect related to injured cells in a product is their possible recovery inside the host gastro-intestinal tract. On consumption of ice cream, containing any injured cells, there is a possibility of

such organisms to reach the target site in the host by withstanding the host's natural barriers against ingested bacteria. We also studied if such injured cells would have the ability to survive and recover in the harsh condition of acidity and bile concentrations commonly encountered in the gastro-intestinal tract of humans. To ascertain this, the injured cell recovery process was also evaluated in simulated gastrointestinal fluids.

MATERIALS AND METHODS

Analysis of Ice Cream Mix Samples

The ice cream mix samples of 42% total solids were obtained from a commercial ice cream manufacturer. The samples were analyzed for standard plate counts, upon receiving by tempering the ice cream mix at 40°C and aseptically drawing 11 grams of the sample in 99 mL of PBS. Further dilutions were made by adding 1 mL of this suspension in 9 mL of PBS and the desired dilutions were plated on Brain Heart Infusion agar for standard plate counts. The selected colonies were further identified using MALDI-TOF as explained before in the earlier chapters. In addition, water activity and pH of the mix was measured using aqua lab CX-2 system (Aqua Lab, Decagon Devices, Inc.), and A321 pH meter (Orion Star^{TM} A321, Thermo Scientific), respectively.

Influence of Background Microflora. In order to study the influence of stages of ice cream manufacture and handling abuse on the background microflora, the samples were analyzed at different stages of ice cream manufacture and three cycles of temperature abuse (Figure 1) as detailed later in this chapter. Standard

methods of analysis were used by taking 11 grams of the samples and suspending in 99 mL of phosphate buffered saline to make the first dilution. Appropriate sequential dilutions were made and plated on BHI agar. The plates were incubated at 37°C for 24 hrs. and the colony counts were expressed as $\log_{10}$ CFU per grams.

Evaluating the Antimicrobial Activity of Background Microflora by Spot on Lawn Assay. The selected colonies form the background microflora were tested for any antagonism against *L. innocua* using 'spot on lawn assay'. In this case, a lawn of *L. innocua* was created by seeding the top layer with soft agar on pre-poured TSA plates. The cell free extracts, obtained from the BHI broth growth of the selected isolates of *Bacillus pumilus* and *Bacillus licheniformis,* were spotted on the seeded layer, and the plates were incubated at 37°C for 24h. At the end of incubation period, the plates were observed for any 'zones of clearance', which were indicative of the antimicrobial activity.

Organism and Culture Conditions

Sourcing Listeria innocua and Propagation. Pure culture of *Listeria innocua* ATCC 33090, (an established surrogate of *L. monocytogenes*) was procured from American Type Culture Collection (Manassas, VA, USA). It was activated in BHI broth (Oxoid, Thermo Scientific, UK) by incubating at 37°C for 24 hours. In order to achieve the mid-exponential cells, the activated culture was sub-cultured in 9 mL of BHI broth, and the 6 h growth was pelleted out and suspended in PBS

to get the desired numbers of cells for inoculation purposes. For long term storage, the pelleted culture was maintained in 1.8-mL cryogenic vials (Copan Diagnostic Inc., Murrieta, CA) that contained sterile beads and glycerol. The vials were stored in a NuAire ultralow deep freezer (NuAire Inc., Plymouth, MN) at −80°C until further use.

Challenge Studies

Tracking Heat-injured Cells during Ice Cream Manufacturing Stages. Ice cream mix samples (42% total solids) were tempered to 40°C in a water bath (Wher and Frank, 2004), and inoculated at the average dose levels of log 4.54±0.13 cfu/grams of *L. innocua* activated culture as explained above. The 6 h grown culture was centrifuged (6000 rpm for 15 min at 4°C), and washed twice with PBS to harvest the cells, which were suspended in phosphate buffered saline and used for spiking 90 gram portions of ice cream mix. The spiked ice cream mix samples were batch pasteurized at 69°C for 30 min (corresponds to 80°C for 25 seconds) (PMO, 2015) in a shaker water bath (Lab companion AAH44063U 17L reciprocal shaking water bath, Cole- Parmer, USA). During heating, the core temperature was continuously monitored using the temperature probe. The samples were then rapidly cooled in an ice bath before further processing and analysis. The pasteurized mix samples were aged at 7°C for 12 hours followed by freezing -4°C (benchtop kitchen aid freezing system (Model KPFD200), hardening at -40°C for 4h, and storage at -18°C. The samples were drawn at each stage and direct plated for intact *Listeria* cells (survivors). The

Listeria specific enrichment protocol was followed to enumerate any injured cells. The selected colonies were further identified using Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) at the Veterinary Science Department, SDSU. Each experimental trial was done in the replicates of three and repeated three times.

Recovery of Survivors using Direct Plating Method*.* For each direct analysis, twenty-five grams of spiked and pasteurized ice cream mix sample were drawn and suspended in 225 mL PBS, followed by further serial dilutions, as necessary. Appropriate dilutions were direct plated on an esculin-based medium, Modified Oxford Agar (MOX) to enumerate the *Listeria* cells that might have survived the heat treatment. The direct plating on Oxford agar likely supports the growth of uninjured populations (Hansen and Knochel, 2001). The plates were incubated at 37°C for 24 hours. The incubated plates were observed for typical *Listeria* colonies, which were small black colonies with a black halo (Park et al., 2013).

Recovery of Heat-injured Cells using the BAM Enrichment Protocol. Additionally, in order to enumerate any heat-injured cells of *Listeria,* an enrichment protocol was used as recommended by USFDA (Bacteriological Analytical Manual, BAM, 2017). A 25 grams portion each of the lab pasteurized spiked sample was suspended in 225 mL of buffered *Listeria* Enrichment Broth (BLEB), and incubated for 4 hours at 30°C. *Listeria* selective enrichment supplements (Acriflavin HCl, Nalidixic acid and Cycloheximide) were added after four hours and the samples were further incubated at 30°C. At 24 to 48 h intervals, BLEB enrichments were streaked on MOX agar and a chromogenic

selective medium (RLM) to show the recovery of heat-injured cells. The typical *Listeria* colonies on MOX appeared black with black zones, and on RLM, the colonies were white, with or without halo. The results were recorded as positive or negative, based on the presence or absence of colonies on the selective media used for streaking, due to the recovery of heat-injured cells of *Listeria*.

Simulated Studies to demonstrate the Influence of Ice cream Temperature Abuse on the Recovery of Injured Cells. The spiked samples of ice cream were temperature abused to simulate the consumer handling conditions on the recovery potential of heat-injured cells, if any, using three temperature abuse cycles (Figure 1). The frozen samples were held at 4.4°C in a refrigerator for 12h followed by 30 minutes holding at room temperature (22°C). This was identified as first cycle of temperature abuse. Samples were drawn in triplicates and direct plated on MOX and RLM for any intact cells due to the possible recovery of injured cells. The samples were restored for another 12h at 4.4°C followed by holding at room temperature for 30 minutes before testing for intact cells (considered second cycle of temperature abuse). Finally, the samples were exposed to a third cycle of temperature abuse followed by plating. Such ‘pudding consistency’, is often experienced while serving ice cream in long-term care facilities and nursing homes. Many a times the ice cream is held at refrigeration before serving due to the preference of recovering patients. In addition, there are chances of the unused ice cream samples being held for a couple of days under refrigeration conditions before being served and consumed. Such temperature

abuse conditions may support the recovery process of any carried over heat-injured cells from the ice cream mix stage onwards.

Simulated Studies on the Influence of Gastro-Intestinal Fluids on the Recovery of Injured Cells. To evaluate the survival and recovery of any *Listeria* cells by consuming the ice cream containing any injured cells under the simulated conditions of human upper gastrointestinal transit an *in vitro* protocol was developed by modifying the methods of Mudie et al., (2010).

For studying the transit in the Simulated Gastric Fluid, a simulated gastric fluid (without pepsin, 0.2%(w/v) sodium chloride in 0.7% hydrochloric acid) was purchased from Fisher Scientific (Manufacturer, Ricca Chemical Company, 710816) and adjusted to the gastric fluid pH variability at 1.0, and 2.0 by neutralizing with filter sterilized sodium bicarbonate (10%) solution.

Assuming that US quart is 32 fl. oz, that means that one scoop will contain 2 fl. oz of ice cream, or 1/4 cup or 56.70g. If a person consumed appx. one scoop of ice cream with injured cells, then it will get mixed with about 50mL of gastric fluid (56.70g ice cream vs. 50mL gastric fluid or appx. 1:1 dilution). Keeping this in mind, the experimental design was made and the ice cream (containing injured cells) and the gastric fluids (at different pH values as above) were held at 37°C in a shaker water bath. Based on the gastric emptying time in the range of 5 to 60 min, samples were drawn at 15, 30, and 60 min intervals. The reaction was stopped by increasing the sample pH to 7.5 with IN NaOH, and direct plated on MOX for enumerating any intact cells that may have recovered from injured cells present in ice cream, while being passed through the gastric fluid. Additionally,

the samples were also enriched in BLEB and plated on MOX to confirm the presence of injured cells. The shortest contact time duration that showed intact cells on MOX helped to interpret any recovery potential in gastric fluid, while the longest contact time duration still showing no intact cells on MOX meant that despite exposure in gastric fluid the cells were still injured and did not recover.

For studying the sequential transit in simulated intestinal fluid, the shortest time duration, under each pH treatment, which was negative for intact cells (assumption is that it still has viable injured cells) was selected and exposed to Simulated Intestinal Fluid (without pancreatin, USP XXII formulation) from Fisher Scientific (Manufacturer, Ricca Chemical Comapany, 7109.7516). Considering the average small intestinal fluid to be at pH 6.8 and its volume to be 50mL (after food intake), 2.0 g of gastric fluid + ice cream with injured cells (1:1) from previous experiment was added to 50mL of simulated intestinal fluid and held at 37°C in a shaker water bath. Keeping in mind the small intestine emptying time in the range of 30 to 360min, samples were drawn at 30, and 360 min intervals. Samples were direct plated on MOX for enumerating any intact cells that may have recovered from injured cells present in ice cream, while being passed through the simulated gastric fluid and simulated intestinal fluid. The shortest contact time duration that shows intact cells on MOX helped to interpret any recovery potential of injured cells in the simulated intestinal fluid, while the longest contact time duration still showing no intact cells on MOX meant that despite exposure in simulated gastric fluid followed by simulated intestinal fluid the cells were still injured and did not recover. At the final stage of experiment, a

BLEB enrichment (BAM protocol) was done to confirm the presence of injured cells in the ice cream samples passed through simulated gastric and intestinal fluids.

Statistical Analysis. The results related to plate counts were subjected to analysis of variance (ANOVA) using SAS® 9.3 (SAS Institute Cary, NC, USA). The level of significance was set to 5%.

RESULTS AND DISCUSSION

Changes in Total Microbial Counts of Ice Cream Mix, and Colony Characteristics

The mean background microflora in the raw ice cream was about log 5.23±0.1cfu/grams and the pH and water activity were 6.56 and 0.958±0.10, respectively. This data was comparable to the baseline value as established in our previous trials. This data was also used to compare the SPC counts at later stages of the analysis, i.e. after pasteurization stage, and at the time of temperature abuse cycles (Figure 2). The log counts of the mix after pasteurization were 2.81 cfu/grams, which did not change significantly during temperature abuse cycles. Similarly, the water activity levels remained pretty much unchanged and comparable to the original mix (Table 1).

Listeria inhibition by background microflora

Another interesting observation was that the background microflora of the pasteurized mix predominated with spore formers such as *B. pumilus and B. licheniformis,* as identified by MALDI-TOF. Amongst these, the *B. pumilus*

isolates appeared as mucoid colonies (Figure 3A) from which long string of exopolymeric substances (EPS) could be stretched, as visible in Figure 3B. On the other hand, *Bacillus licheniformis* formed typical spreader colonies, which were non- mucoid (Figure 3C).

Background microflora in product matrices can potentially influence the recovery process of injured cells in the product. It was hypothesized that such a situation could arise for the ice cream containing injured cells also, if the background microflora could directly inhibit the *Listeria* cells. The 'spot on lawn study' conducted using cell free extract of *B. pumilus* and *B. licheniformis* provided an early evidence of the direct inhibition of *Listeria innocua.* This was clearly evident by the zones of clearance produced on the *L. innocua* plates. Figure 4 depicts such a zone of clearance by the CFE of *B. licheniformis.* This is another novel finding and needs further studies to identify the factors responsible for this inhibition of *L. innocua* by the CFE of background microflora, and their role in preventing the potential recovery of injured cells of *Listeria* in the ice cream matrix.

Effect of Ice cream Manufacturing Stages on Listeria Survivors. The ice cream mix samples were spiked at log 4.54±0.13 cfu/grams levels and exposed to minimum pasteurization treatment (69°C for 30 min). The direct plating on MOX and RLM did not detect any *Listeria* survivors. On the other hand, injured cells were recovered by using the BLEB enrichment protocol followed by plating on MOX and RLM. These results show the effectiveness of pasteurization in eliminating intact *Listeria* and is similar to our previous findings (Neha eta al.,

2018) and several others (Bradshaw et al., 1987, Holsinger et al., 1992). Direct plating technique does not recover injured or stressed cells (Jasson et al., 2010), hence by following the enrichment protocol, we detected a random presence of some heat-injured cells. This may be due to the sub lethal damage of cytoplasmic membrane, also known as structural damage (Hauben et al., 1996; Pagan and Mackey, 2000 and Ritz et al., 2001) or damage to intracellular components, also known as physiological damage (Niven et al., 1999 and Ritz et al., 2002). In order to establish any potential risk due to injured cells recovery in product matrix, especially for the higher risk population including immunocompromised, elderly people, and pregnant women (Choi et al., 2016), recovery of injured cells was tracked during the ice cream manufacturing stages such as freezing, hardening, and storage. Similar to our previous findings (Neha eta al, 2018), which depicted no recovery of injured cells in ice cream mix holding conditions, none of the later manufacturing stages supported the recovery of any injured cells. This study thus provided evidence that despite the random presence of injured cells, the ice normal ice cream manufacturing and storage conditions do not offer any risk from the recovery of any heat-injured cells that are carried over to the final product. This is an important finding that further supports the view that post pasteurization environmental contamination of the finished product is more critical in establishing the *Listeria* risk in a more realistic manner.

Effect of Product Temperature Abuse on Recovery Potential of Injured Cells. The next part of the study evaluated the role of temperature abuse at the

consumer end on the recovery of any carried over injured cells in the final product. From the consumer perspective it was observed that in nursing homes and patient care facilities the ice cream is held at the refrigerated temperature overnight for tempering, before serving to the patients. This may result in temperature abuse of ice cream. To simulate the long-term care facilities, and old –age nursing homes, the ice cream samples with injured *Listeria* cells were exposed to refrigeration temperature storage cycles. Such storage resulted in 'pudding like consistency' to the ice cream samples, during the overnight temperature abuse cycles. Direct plating was done on MOX at the end of each of the three cycles, and it was observed that none of the temperature abuse cycles resulted in any recovery of any carried over injured cells. On the other hand, the BLEB enriched samples followed by MOX plating revealed the presence of injured cells. This helped us to reiterate that despite the random presence of injured cells in the final product, none of the later stages of normal ice cream holding or refrigerated temperature abuse resulted in the recovery of those in injured cells. Such an evidence will go a long way in developing more robust risk assessment models, and help explain the causes of any *Listeria* outbreaks, from products such as ice cream, in a more realistic manner.

All this information further points towards the necessity of directing greater efforts in environmental *Listeria* control to prevent any post pasteurization contamination of the product with intact cells of *Listeria.* Our further studies about the post pasteurization contamination with intact cells of *Listeria* showed that none of the later stages of freezing, hardening, and storage had any detrimental effect on

Listeria intact cells (data not included). Such intact cells, which cause environmental cross contamination of the finished product, are likely to be more significant in causing any disease outbreaks and need to be monitored and controlled more effectively.

Potential of Injured Cells Recovery in the Simulated Gastro-intestinal Fluids. Many a times a question comes up that if the injured cells are consumed along with a cross-contaminated product, would they have the tolerance to gastro-intestinal fluids and cause a disease. There are not many studies conducted to evaluate this effect, hence, we conducted a simulated study to observe the recovery potential of injured cells of *Listeria* in the ice cream when exposed to GI fluids.

The two-stage experiment was conducted by exposing the ice cream samples, containing injured cells, to simulated gastric and intestinal fluids in a sequential manner. The injured cells were detected on exposure to the gastro-intestinal fluids using BLEB enrichment only. The direct plating on MOX did not detect any cells, indicating the inability of injured cells to recover during the exposure to gastro-intestinal fluid, simulating the gastric transit in the host (Table 2). However, it is also to be noted that the injured cells remained injured in the simulated gastro-intestinal fluid passages and even the low pH of gastric fluid was not sufficiently effective in eliminating them completely. This study has a limitation of being conducted under *in vitro* conditions, in the absence of any gut microbiome. However, it provides an early evidence that it is not likely that these injured cells would recover when exposed to gastro-intestinal fluids. Further

studies need to be conducted under better simulated gut microbiome models, and appropriate animal model to arrive at final conclusions.

CONCLUSIONS

This study evaluates the recovery potential of injured cells of *Listeria innocua* in ice cream matrix under different condition of manufacturing and storage. The results provided an evidence about the inability of the randomly presented injured cells to recover under later manufacturing steps of freezing, hardening and storage. Even the refrigerated temperature abuse cycles, assuming a consumer end mishandling of product, also did not show the recovery of any injured cells. Further studies conducted using simulated gastro-intestinal fluids did not provide any evidence of the recovery of injured cells. This study thus confirms that any randomly present injured cells in ice cream mix are not likely to recover during the later stages of ice cream manufacturing or storage. The simulated gastro-intestinal studies provide any early indication on the inability of injured cells to recover in the host system but would require more in-depth gut microbiome models to clearly establish this finding.

Chapter 5 Tables and Figures.

Table 1. Changes in water activity and pH of ice cream mix and different stages.

	Water activity	**pH**
Raw ice cream mix	0.958	6.56
Post pasteurization	0.961	6.57
Temp abuse Cycle 1	0.969	6.63
Temp abuse Cycle 2	0.981	6.65
Temp abuse Cycle 3	0.985	6.62

Table 2. Recovery of injured cells in gastro-intestinal fluids.

Test medium	**Exposure time (min)**	**Direct Plating on MOX**	**BLEB Enrichment and plating on MOX and RLM**
Gastric fluid pH 1.0 and 2.0	15	-* -	+ +
	30	- -	- +
	60	- -	+ -
Intestinal fluid pH 6.8	30	- -	+ -
	360	- -	+ +

*(-) Not detected

Figure 1. Flowchart for the temperature abuse cycles of ice cream.

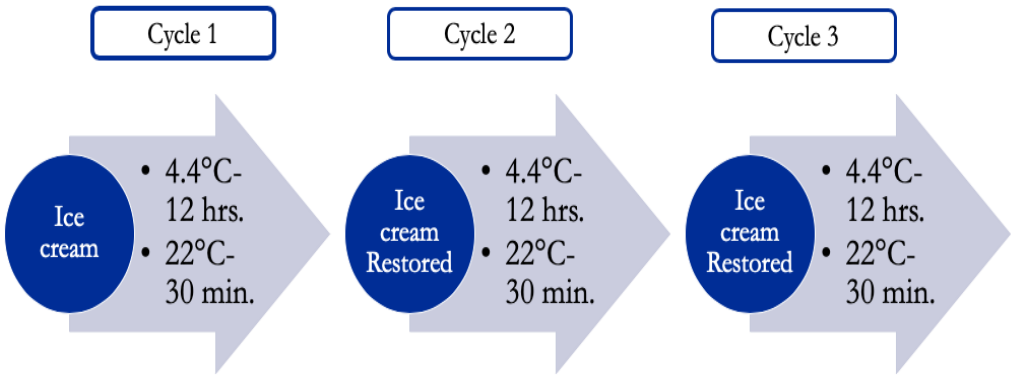


Figure 2. Standard plate counts (log cfu/g) of ice cream mix samples at different stages of treatment and temperature abuse (cycles 1 to 3).

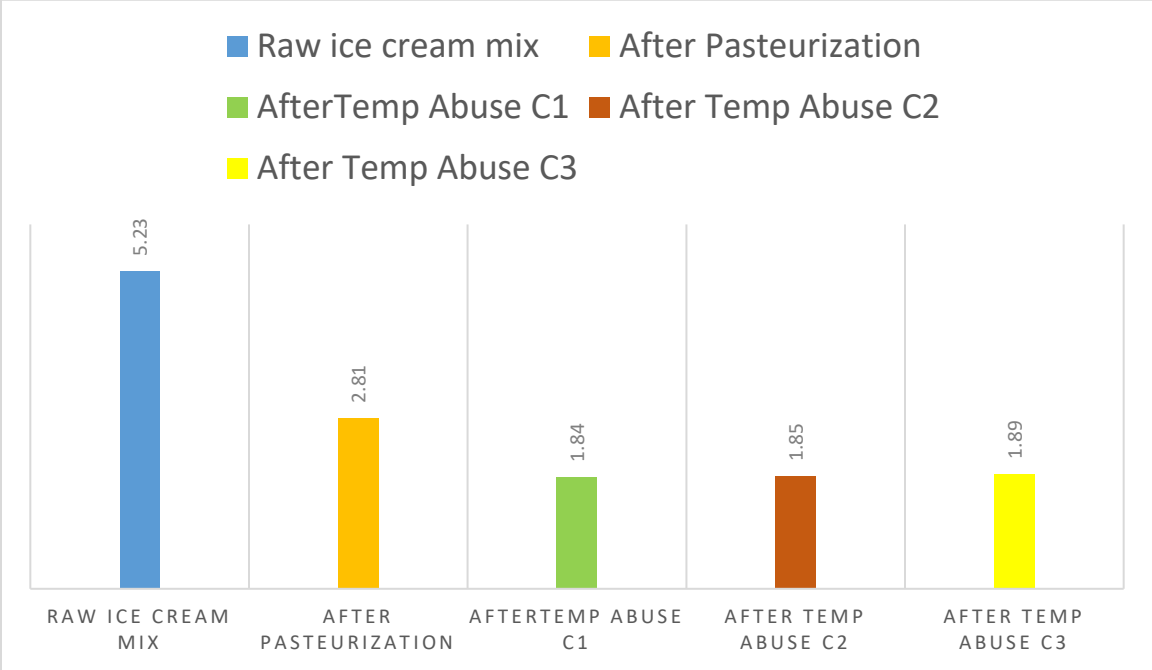
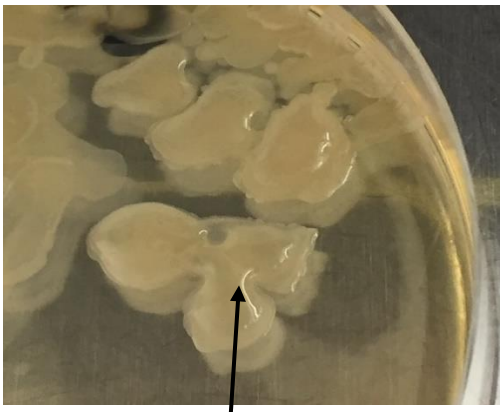


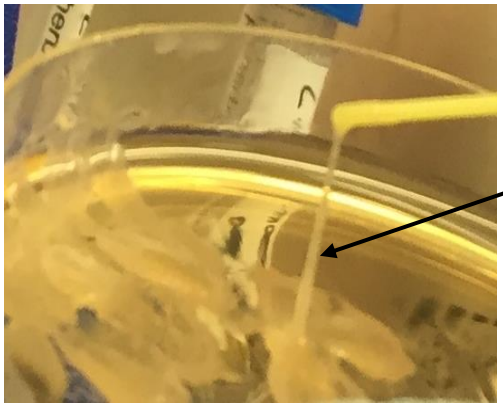
Figure 3. Colony characterization of the background microflora in the post pasteurized ice cream, mix samples. Mucoid colonies of *Bacillus pumilus* (3A) and EPS string of *Bacillus pumilus* colony (3B), spreader non-mucoid colony of *Bacillus licheniformis.*

A



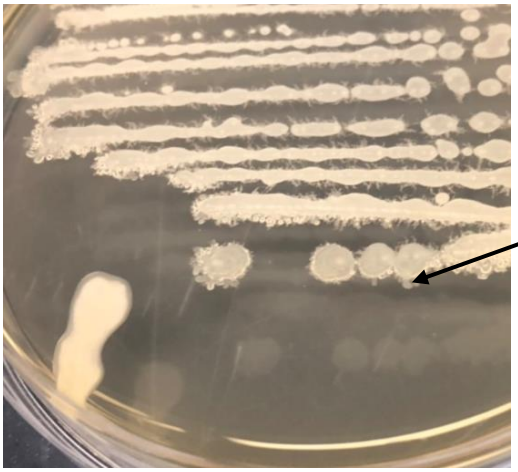
Mucoid colony

B



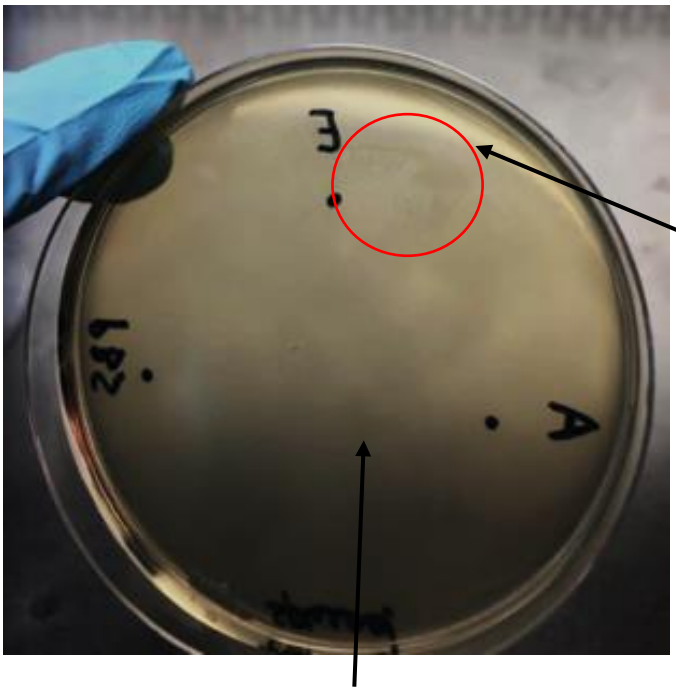
EPS String

C



Non-Mucoid colony

Figure 4. Spot on lawn assay showing zones of clearance of the growth of *L. innocua* when spotted with the cell free extract of *B. licheniformis.*



SUMMARY AND CONCLUSIONS

Phase I of the study focused on the relationship of cross contamination levels at raw ice cream mix stage and the presence and recovery potential of heat-injured cells using enrichment protocols. When the raw ice cream mix samples were inoculated with about log 4.0 cfu/g of *Listeria innocua* (an established surrogate) and heat treated to minimum pasteurization treatment, the direct plating did not detect any survivors. On the other hand, BLEB enrichment protocol detected a random presence of heat-injured cells. Statistical algorithms were used to fit a model by considering covariates. Based on the quasi- complete separation, dose (level of cross contamination) came up as a predictor of the presence of heat-injured cells only at the higher levels of cross contamination (average log 4.0 or more). Further studies are necessary to evaluate the significance of the possibilities of random presence of heat-injured cells in ice cream mix when subjected to minimum pasteurization treatment. Importantly, ice cream mix itself did not support the recovery of any heat-injured cells during common aging and storage conditions (7°C for 72 h), whereas the influence of any abused handling conditions is yet to be studied. Many ice cream-related outbreaks have occurred in the recent past, mostly due to cross contamination under unhygienic processing conditions. *Listeria* is a very resilient organism that can withstand adverse conditions, such as wide ranges of pH, water activity, and low temperatures. Its persistence in the dairy processing environment has been reported, as the organism can form resistant biofilms leading to potential cross contamination of the product, especially from harborage sites. Its ability to

withstand adverse processing conditions may also lead to stressed and injured cells of *Listeria*, which may recover under abused conditions of handling and storage. Although such injured cells have not been linked to any disease outbreaks so far, this information could be helpful in improving risk assessment protocols.

Phase II of the study suggested a possible role of air-pocket entrapment in protecting *Listeria* cells by limiting their exposure to the complete thermal effect. This information thus provides another perspective around *Listeria* risk, in addition to the other reported factors such as higher cross contamination levels, background microflora, product constituents, lipid-shielding effects, and entrapment in polymorphonucleocytes. We hope that this additional information about air cell entrapment may serve to develop more robust *Listeria* risk-assessment protocols by considering the potential presence of heat-injured cells. This is an early evidence proposing the implications of air cells entrapped *Listeria* on their later detection as heat-injured cells in the heat-treated viscous food matrices such as ice cream mix. Further studies are necessary to evaluate the complete extent of these findings and their relationship to any injured cells detection.

Phase III of the study evaluated the recovery potential of injured cells of *Listeria innocua* in ice cream matrix under different condition of manufacturing and storage. The results provided an evidence about the inability of the randomly presented injured cells to recover under later manufacturing steps of freezing, hardening and storage. Even the refrigerated temperature abuse cycles,

assuming a consumer end mishandling of product, also did not show the recovery of any injured cells. Further studies conducted using simulated gastro-intestinal fluids did not provide any evidence of the recovery of injured cells. This study thus confirms that any randomly present injured cells in ice cream mix are not likely to recover during the later stages of ice cream manufacturing or storage. The simulated gastro-intestinal studies provide any early indication on the inability of injured cells to recover in the host system but would require more in-depth gut microbiome models to clearly establish this finding.

LIST OF REFERENCES

Alessandria, V., K. Rantsiou, P. Dolci, and L. Cocolin. 2010. Molecular methods to assess *Listeria monocytogenes* route of contamination in a dairy processing plant. International Journal of Food Microbiology. 141 (Suppl 1): S156-162.

Azizoglu, R.O., J. Osborne, S. Wilson, and S. Kathariou. 2009. Role of Growth Temperature in Freeze-Thaw Tolerance of *Listeria* spp. Applied and Environmental Microbiology, 75:5315–5320.

Bearns, R.E., and K.F. Girard. 1958. The effect of pasteurization on *Listeria monocytogenes.* Canadian Journal of Microbiology. 4:55–61.

Besse, N. 2002. Influence of various environmental parameters and of detection procedures on the recovery of stressed *L. monocytogenes*: a review. Food Microbiology. 19:221-234.

Boyle, D.L., Sofos, J.N. and Schmidt, G.R. 1990. Thermal destruction of *Listeria monocytogenes* in a meat slurry and in ground beef. *Journal of Food Science*. 55(2), 327-329.

Bradshaw, J.G., J.T. Peeler, J.J. Corwin, J.M. Hunt, and R.M. Twedt. 1987. Thermal resistance of *Listeria monocytogenes* in Dairy Products. Journal of Food Protection. 50:543–544.

Buchanan, R.L. 1990. Advances in culture methods for detection of *Listeria monocytogenes.* P. 85-95. *In* A.J. Miller, J.L. Smith. And S.A. Somkuti (eds.), Foodborne Illness. Society for Industrial Microbiology, Washington, D.C.

Buchanan, R.L., L.G. Gorris, M.M. Hayman, T.C. Jackson, and R.C. Whiting. 2017. A review of Listeria monocytogenes: An update on outbreaks, virulence, dose-response, ecology, and risk assessments. *Food Control*. 75:1–13.

Bunduki, M.M.-C., K.J. Flanders, and C.W. Donnelly. 1995. Metabolic and Structural Sites of Damage in Heat- and Sanitizer-Injured Populations of *Listeria monocytogenes*. Journal of Food Protection. 58:410–415.

Bunning, V. K., C.W. Donnelly, J.T. Peeler, E.H. Briggs, J.G. Bradshaw, R.G. Crawford, C.M. Beliveau, and J.T. Tierney. 1988. Thermal inactivation of Listeria monocytogenes within bovine milk phagocytes. Applied and Environmental Microbiology, 54(2), 364–370.

Busta, F.F. 1976. Practical implications of injured microorganisms in food. J. Milk Food Technol. 39(2), 138-145.

Busta, F.F. 1978. Introduction to injury and repair of microbial cells. In Advances in Applied Microbiology, Vol. 23. (D. Perlman, ed.) pp. 195-201, Academic Press, New York.

Campbell, M. 2015. An examination of microbial populations in different brands and flavors of Ice Cream. Undergraduate thesis, University of Mississippi.

Centers for Disease Control and Prevention (CDC). Listeriosis disease information (online). CDC; 2005 Available at: (www.cdc.gov/ncidod/dbmd/diseaseinfo/listeriosis_t. htm, 2005. Accessed 11 Nov. 2017.

Centers for Disease Control and Prevention. 2015. Multistate outbreak of Listeriosis linked to Blue Bell Creameries products (Final Update). Retrieved from https://www.cdc.gov/listeria/outbreaks/ice-cream-03-15/index.html

Chen, Y., L.S., Burall, D.M. Re´Gis Pouillot, E. Strain, A.J. De Jesus, A. Laasri, H. Laila Ali, A. Tatavarthy, G. Zhang, L. Hu, J. Day, J. Kang, S. Sahu, D. Srinivasan, K. Klontz, M. Parish, P. S. Evans, E.W. Brown, T. S. Hammack, D.L. Zink, and A.R. Datta. 2016. Prevalence and Level of *Listeria*

monocytogenes in Ice Cream Linked to a Listeriosis Outbreak in the United States. Journal of Food Protection, 79:1828–1832.

Choi, K.H., H. Lee, S. Lee, S. Kim, and Y. Yoon. 2016. Cheese microbial risk assessments — A Review. Asian-Australasian Journal of Animal Sciences. 29:307–314.

Codex Alimentarius Commission. 1999. Principles and Guidelines for the Conduct of Microbiological *Risk Assessment*. (CAC/GL 30 – *1999*): 1-15.

Cotton, L. N., and C. H. White. 1992. *Listeria monocytogenes, Yersinia enterocolitica, and Salmonella* in dairy plant environments. *Journal of Dairy Science*. 75:51–57.

Dean, J. P., and E. A. Zottola. 1996. Use of nisin in ice cream and effect on the survival of *Listeria monocytogenes*. *Journal of Food Protection.* 59:476–480.

Doyle, M.P. 1985. Foodborne pathogens of recent concern. Annual Review of Nutrition. 5, 32-34.

Doyle M. P, Glass K. A, Beery J.T., Garcia G.A., Pollard D.J, Schultz R.D. 1987. Survival of *Listeria monocytogenes* in milk during high-temperature,

short-time pasteurization. Applied and Environmental Microbiology. 53(7):1433-1438.

Dykes, G.A. 1999. Physical and metabolic causes of sub-lethal damage in *Listeria monocytogenes* after long-term chilled storage at 4 °C. Journal of Applied Microbiology 1999, 87, 915–922.

El-Kest, S.E., and E.H. Marth. 1992. Freezing of Listeria monocytogenes and Other Microorganisms: A Review. *Journal of Food Protection*. 55:639–648.

Environmental Protection Agency. 2012. Microbial risk assessment guideline: Pathogenic microorganisms with focus on food and water. U.S. Environmental Protection Agency; U.S. Department of Agriculture, Food Safety and Inspection Service, Washington, D.C.

Fan, W., Bahrami, B., Chen, K., Mabrouk, S., Pathak, R., Tong, Y., Li, X., Zhang,T., Jian, R., and Qiao.Q. 2018. Bias-Dependent Normal and Inverted J–V Hysteresis in Perovskite Solar Cells. ACS Applied Materials & Interfaces 10(30):25604-25613.

Fanelli, D., and McKee, M. 2007. Microbes can survive ‘deep freeze’ for 100,000 years. New Scientist, Daily News Oct 2007. Retrieved from

https://www.newscientist.com/article/dn12752-microbes-can-survive-deep-freeze-for-100000-years/, accessed on 04-04-2018).

Farber, J., G. Sanders, J. Speirs, J.-Y. Daoust, D. Emmons, and R. Mckellar. 1988. Thermal resistance of *Listeria monocytogenes* in inoculated and naturally contaminated raw milk. International Journal of Food Microbiology. 7:277-286.

Farber, J., and Peterkin, P. 1991. *Listeria monocytogenes,* a food-borne pathogen. American Society of Microbiology. 55:476-511.

Farber, J., E Dailey, D.B. Emmons, and R. Mckellar. 1992. Factor influencing survival of *Listeria monocytogenes* in milk in a high-temperature short-time pasteurizer. Journal of Food Protection. 55:946-951.

Fernandez Garayzabal, J.F.F., L.D. Rodriguez, J.A.V. Boland, E.F.R. Ferri, V.B. Dieste, J.L.B. Cancelo, and G.S. Fernandez. 1987. Survival of *Listeria monocytogenes* in raw milk treated in a pilot plant size pasteurizer. Journal for Applied Bacteriology. 63:533-537.

Ferreira, V., M. Wiedmann, P. Teixeira, and M.J. Stasiewicz. 2014. Listeria monocytogenes Persistence in Food-Associated Environments:

Epidemiology, Strain Characteristics, and Implications for Public Health. *Journal of Food Protection*. 77:150–170.

Flanders, K. J., T. J. Pritchard, and C. W. Donnelly. 1994. Enhances recovery of *Listeria* from dairy-plant processing environments through combined use of repair enrichment and selective enrichment/ detection procedures. Journal of Food Protection 58:404–409.

Fleming, D., S. Cochi, and K. Macdonald. 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. American Journal of Infection Control. 13:284–285.

Fleming, D.W., S.L. Cochi, K.L. MacDonald, J. Brondum, S. P.S. Hayes, B.D. Pllikaytis, M.B. Holmes, A. Audurier, C.V. Broome, and A.L. Reingold. 1985. Pasteurized milk as a vehicle of infection in an outbreak of Listeriosis. New England Journal of Medicine. 312:404-407.

Fleming, D., S. Cochi, and K. Macdonald. 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. American Journal of Infection Control. 13:284–285.

Fleming, D.W., S.L. Cochi, K.L. MacDonald, J. Brondum, S. P.S. Hayes, B.D. Pllikaytis, M.B. Holmes, A. Audurier, C.V. Broome, and A.L. Reingold. 1985.

Pasteurized milk as a vehicle of infection in an outbreak of Listeriosis. New England Journal of Medicine. 312:404-407.

Fleming, D., S. Cochi, and K. Macdonald. 1985. Pasteurized milk as a vehicle of infection in an outbreak of listeriosis. American Journal of Infection Control. 13:284–285.

Foegeding, P.M. And Ray, B. 1992. Repair and detection of injured microorganisms. In Compendium of Methods for the Microbiological Examination of Foods. 3rd Ed. (C. Vanderzant and D.F. Splittstoesser, eds.) pp. 121-134, American Public Health Association, Washington, DC.

FDA. 2015. Pasteurized milk ordinance. US Department of Health and Human Services, Public Health Service, Food and Drug Adminis- tration, Silver Spring, MD.

FDA. 2016. Jeni's Splendid Ice Creams, LLC 8/9/16. Accessed Aug. 15, 2018. https://www.fda.gov/iceci/enforcementactions/ warningletters/2016/ucm516395.htm.

FDA. 2017. Detection of *Listeria monocytogenes* in foods and en- vironmental samples, and enumeration of listeria monocyto- genes in foods. Chapter 10 in Bacteriological Analytical Manual (BAM). Accessed Aug. 15, 2018.

https://www.fda.gov/food/
foodscienceresearch/laboratorymethods/ucm071400.htm.

FDA. 2018. Fieldbrook Foods Corporation, announces an extension of voluntary recall of orange ice cream bars and chocolate coated vanilla ice cream bars for possible health risk. Retrieved from https://www.fda.gov/Safety/Recalls/ucm591923.htm (accessed on April 12, 2018).

Fox, E., T. Omahony, M. Clancy, R. Dempsey, M. Obrien, and K. Jordan. 2009. *Listeria monocytogenes* in the Irish dairy farm environment. Journal of Food Protection. 72:1450–1456.

Friedly, E. c., P.G. Crandall, S. Ricke, C.A. O'Bryan, E.M. Martin, and L.M. Boyd. 2008. Identification of *Listeria innocua* surrogates for *Listeria monocytogenes* in hamburger patties. Journal of Food Science. 73: M174-178.

FSIS. 2014. Compliance guideline: Controlling *Listeria monocytogenes* in post-lethality exposed ready-to -eat meat and poultry products. Accessed Aug. 15, 2018. https://www.fsis.usda.gov/wps/wcm/ connect/d3373299-50e6-47d6-a577-e74a1e549fde/Controlling-Lm -RTE-Guideline.pdf ?MOD=AJPERES.

Fung, D.Y.C., and L.L.V. Bosch. 1975. Repair, Growth, and Enterotoxigenesis of Staphylococcus aureus S-6 Injured by Freeze-Drying. *Journal of Milk and Food Technology*. 38:212–218.

Glass, K.A. and Doyle, M.P. 1989. Fate and thermal inactivation of Listeria monocytogenes in beaker sausage and pepperoni. Journal of Food Protection. 52(4):226-231.

Glicksman, M. 1983. Red seaweed extracts (agar, carrageenans, furcellaran). Chap. 5. Pp. 73-113. *In M. Glicksman (ed.),* Food Hydrocolloids, vol II. CRC Press, Boca Raton, FL.

Goff H.D, 2018, The Ice cream ebook, Univeristy of Guleph. Retrieved from https://www.uoguelph.ca/foodscience/book-page/ice-cream-manufacture.

Gougouli, M., A. Angelidis, and K. Koutsoumanis. 2008. A study on the kinetic behavior of *Listeria monocytogenes* in Ice Cream stored under static and dynamic chilling and freezing conditions. Journal of Dairy Science. 91:523–530.

Hansen, T.B., and S. Knøchel. 2001. Factors influencing resuscitation and growth of heat injured *Listeria monocytogenes* in sous vide cooked beef. International Journal of Food Microbiology. 63:135–147.

Hassan, A., S. Anand, and M. Avadhanula. 2010. Microscopic observation of multispecies biofilm of various structures on whey concentration membranes. Journal of Dairy Science. 93:2321–2329.

Hauben, K.J.A., E.Y. Wuytack, C.C.F. Soontjens, and C.W. Michiels. 1996. High-pressure transient sensitization of *Escherichia coli* to lysozyme and nisin by disruption of outer-membrane permeability. *Journal of Food Protection. 59:350–355.*

Heiman KE, Garalde VB, Gronostaj M, Jackson KA, Beam S, Joseph L, et al. Multistate outbreak of listeriosis caused by imported cheese and evidence of cross-contamination of other cheeses, USA, 2012. Epidemiol Infect. 2016; 144:2698–708.

Highmore, C.J., J.C. Warner, S.D. Rothwell, S.A. Wilks, and W Keevil. 2018. Viable-but-nonculturable *Listeria monocytogenes* and *Salmonella enterica* Serovar Thompson induced by chlorine stress remain infectious. mBio 9:e00540-18. https://doi.org/10.1128/mBio.00540-18.

Hitchens, A.D., K. Jinneman, and Y. Chen. Detection of *Listeria monocytogenes* in Foods and Environmental Samples, and Enumeration of *Listeria monocytogenes* in Foods. Chapter 10. Bacteriological Analytical Manual, FDA. Retrieved from https://www.fda.gov/Food/FoodScienceResearch/LaboratoryMethods/ucm071400.htm

Hoffmans, C.M., D.Y.C. Fung, and C.L. Kastner. 1997. Methods *and* resuscitation environments for the recovery of *Listeria monocytogenes* a review. Journal of Rapid Methods and Automation in Microbiology 5 (1997) 249-268.

Holsinger, V. H., P.W. Smith, J.L. Smith, and S.A. Palumbo. 1992. Thermal destruction of *Listeria monocytogenes* in ice cream mix. Journal of Food Protection. 55: 234-237.

Hunt, K N. Drummond, M. Murphy, F. Butler, J. Buckley, and K. Jorda. 2012. A case of bovine raw milk contamination with *Listeria monocytogenes*. Irish Veterinary Journal, 65:13.

Jasson, V., L. Jacxsens, P. Luning, A. Rajkovic, and M. Uyttendaele. 2010. Alternative microbial methods: An overview and selection criteria. *Food Microbiology*. 27:710–730.

Johnsen, B.O., E. Lingaas, D. Torfoss, E.H. Strøm, and I. Nordøy. 2010. A large outbreak of Listeria monocytogenes infection with short incubation period in a tertiary care hospital. *Journal of Infection*. 61:465–470.

Knable, S.J., H.W. Walker, P.A. Hartman, and A.F. Mendonca/ 1990. Effects of growth temperature and strictly anaerobic recovery of on the survival of *Listeria monocytogenes* during pasteurization. Applied Environmental Microbiology. 56:370-376.

Kozempel, M., N. Goldberg, E. R. Radewonuk, O. J. Scullen, and J. C. Craig. 2000. Rapid hot dog surface pasteurization using cycles of vacuum and steam to kill listeria innocua. Journal of Food Protection, 63: 457–461.

Li. M, A. Pradhan, W. Wang, and Y. Li. 2013. Prediction of *Listeria innocua* survival in fully cooked chicken breast products during postpackage thermal treatment. Poultry Science 92 :827–835.

Linnan, M.J., L. Mascola, X.D. Lou, V. Goulet, S. May, C. Salminen, D.W. Hird, M.L. Yonekura, P. Hayes, R. Weaver, A. Audurier, B.D. Plikaytis, S.L. Fannin, A. Kleks, and C.V. Broome. 1988. Epidemic Listeriosis Associated with Mexican-Style Cheese. *New England Journal of Medicine*. 319:823–828.

Liu, S., Puri, V. M., and Demirci, A. 2009. Evaluation of *Listeria innocua* as a suitable indicator for replacing *Listeria monocytogenes* during ripening of Camembert cheese. International journal of food science & technology. 44:1:29-35.

Lopez, V., S. Ortiz, A. Corujo, P. Lopez, D. Poza, J. Navas, R. Moreno, and J.V. Martinez-Suarez. 2008. Different Contamination Patterns of Lineage I and II Strains of Listeria monocytogenes in a Spanish Broiler Abattoir. *Poultry Science*. 87:1874–1882.

Lou, Y., and A. E. Yousef. 1999. Characteristics of *Listeria monocytogenes* important to food processors. Pages 131–225 in *Listeria*, Listeriosis, and Food Safety. 2nd ed. E. T. Ryser, and E. H. Marth, ed. Marcel Dekker, New York, NY.

Lovett, J. and Twedt, R.M. 1988. *Listeria*. Food Technol. 42(4), 188-191.

Lovett, J. 1989. *Listeria monocytogenes. In Foodborne Bacterial Pathogens.* (M.P. Doyle, ed.) pp: 284-310, Marcel Dekker, New York.

Lund, A. M., E. A. Zottola, and D. J. Pusch. 1991. Comparison of methods for isolation of *Listeria* from raw milk. Journal of Food Protection. 54:602–606.

Lundén, J.M., T.J. Autio, A.-M. Sjöberg, and H.J. Korkeala. 2003. Persistent and Nonpersistent Listeria monocytogenes Contamination in Meat and Poultry Processing Plants. *Journal of Food Protection*. 66:2062–2069.

Mackey, B., E. Boogard, C. Hayes, and J. Baranyi. 1994. Recovery of heat-injured *Listeria monocytogenes.* International Journal of Food Microbiology. 22:227–237.

Marka, S., and S. Anand. 2018. Feed substrate influence biofilm formation on reverse osmosis membranes and their cleaning efficiency. Journal of Dairy Science. 101:84-95.

Mcdonald, L.C., Hackney, C.R. and Ray, B. 1983. Enhanced recovery on injured Escherichia coli by compounds that degrade hydrogen peroxide or block its formation. Appl. Environ. Microbiol. 45(2), 360-365.

McMahon, C., C. Byrne, J. Sheridan, D. Mcdowell, I. Blair, and T. Hegarty. 2000. The effect of culture growth phase on induction of the heat shock response in *Yersinia enterocolitica* and *Listeria monocytogenes*. Journal of Applied Microbiology. 89:198-206.

Mcclure, P., T. Roberts, and P.O. Oguru. 1989. Comparison of the effects of sodium chloride, pH and temperature on the growth of Listeria

monocytogenes on gradient plates and in liquid medium. *Letters in Applied Microbiology*. 9:95–99.

McKellar, R. 1996. Influence of ice-cream mix components on the thermal stability of bovine milk γ-glutamyl transpeptidase and *Listeria innocua*. International Dairy Journal. 6:1181–1189.

Mead, P.S., L. Slutsker, V. Dietz, L.F. Mccaig, J.S. Bresee, C. Shapiro, P.M. Griffin, and R.V. Tauxe. 1999. Food-Related Illness and Death in the United States. *Emerging Infectious Diseases*. 5:607–625.

Meyer, D.H. and Donnelly, C.W. 1992. Effect of incubation temperature on repair of heat-injured *Listeria* in milk. J. Food Prot. 55(8), 579-582.

Miettinen, M. 1999. Characterization of Listeria monocytogenes from an ice cream plant by serotyping and pulsed-field gel electrophoresis. *International Journal of Food Microbiology*. 46:187–192.

Modified Oxford Agar Base - Thermo Fisher Scientific. Retrieved from https://assets.thermofisher.com/TFS-Assets/LSG/manuals/IFU1613.pdf

Mudie, D.M., G.L. Amidon, and G.E. Amidon. 2010. Physiological parameters for oral delivery and in vitro testing. Molecular Pharmacy. 7: 1388–1405.

Neha, N., S. Anand, G. Djira, B. Kraus, and S. Sutariya. 2018. Listeria cross contamination levels in raw ice cream mix can serve as a predictor of their potential presence as heat-injured cells. *Journal of Dairy Science*. 101:9659–9669.

Nightingale, K.K., Y.H. Schukken, C.R. Nightingle, E.D. Fortes, A.J. Ho, Z. Her, Y.T. Grohn, P.L. Mcdonough, and M. Weidmann. 2004. Ecology and transmission of *Listeria monocytogenes* infecting ruminants and in the farm Environment. Applied and Environmental Microbiology. 70:4458-4467.

Niven, G.W., C.A. Miles, and B.M. Mackey. 1999. The effects of hydrostatic pressure on ribosome conformation in *Escherichia coli*: an in vivo study using differential scanning calorimetry. Microbiology. 145:419–425.

Norton, D.M., M.A. Mccamey, K.L. Gall, J.M. Scarlett, K.J. Boor, and M. Wiedmann. 2001. Molecular Studies on the Ecology of Listeria monocytogenes in the Smoked Fish Processing Industry. *Applied and Environmental Microbiology*. 67:198–205.

Novak, J.S., and V.K. Juneja. 2001. Detection of heat injury in *Listeria monocytogenes* Scott A. Journal of Food Protection. 64:1739–1743.

O’ Donnell, E.T. 1995. The incidence of *Salmonella* and *Listeria* in raw milk from farm bulk tanks in England and Wales. International Journal of Dairy Technology. 48:25–29.

Ottesen, A., P. Ramachandran, E. Reed, J. R. White, N. Hasan, P. Subramanian, G. Ryan, K. Jarvis, C. Grim, N. Daquiqan, D. Hanes, M. Allard, R. Colwell, E. Brown, and Y. Chen. 2016. Enrichment dynamics of *Listeria monocytogenes* and the associated microbiome from naturally contaminated ice cream linked to a listeriosis outbreak. BMC Microbiology.16:275.

Pagan, R., and B. Mackey. 2000. Relationship between membrane damage and cell death in pressure-treated *Escherichia coli cells*: Differences between exponential and stationary phase cells and variation among Strains. Applied and Environmental Microbiology. 66:2829–2834.

Palumbo, S. A., and A. C. Williams. 1991. Resistance of *Listeria monocytogenes* to freezing in foods. Food Microbiol. 8:63–68.

Park, S. H., P.S. Chang, S. Ryu, and D.-H. Kang. 2013. Development of a Novel Selective and differential medium for the isolation of *Listeria monocytogenes*. Applied and Environmental Microbiology. 80:1020–1025.

Pascual, C., T.P. Robinson, M.J. Ocio, O.O. Aboada, and B.M. Mackey. 2001. The effect of inoculum size and sulethal injury on the ability of *Listeria monocytogenes* to initiate growth under suboptimal conditions. Letters in Applied Microbiology. 33: 357-361.

Pasteurized Milk Ordinance (2015 revision). U.S. department of health and human services/ Public health service/ food and drug administration.

Pearson, L.J. and Marth, E.H. 1990. *Listeria monocytogenes* - threat to a safe food supply: A review. J. Dairy Sci. 73, 912-928.

Pérez-Trallero E, Zigorraga C, Artieda J, Alkorta M, Marimón JM. Two outbreaks of *Listeria monocytogenes* infection, northern Spain. Emerging Infectious Disease. 2014; 20:2155–7.

Petran, R.L., and E.A. Zottola. 1989. A Study of Factors Affecting Growth and Recovery of Listeria monocytogenes Scott A. *Journal of Food Science*. 54:458–460.

Phan-Thanh, L., and A. Montagne. 1998. Physiological and biochemical aspects of the acid survival of Listeria monocytogenes. *The Journal of General and Applied Microbiology*. 44:183–191.

Pouillot, R.C.A., K.C. Klontz, Y. Chen, L.S. Burall, D. Macarisin, M. Doyle, K.M. Bally, E. Strain, A.R. Datta, T.S. Hammack, and J.M.V. Doren. 2016. Infectious dose of *Listeria monocytogenes* in outbreak linked to ice cream, United States, 2015. Emerging Infectious Diseases. 22:2113–2119.

Pritchard, T.J., and C.W. Donnelly. 1999. Combined secondary enrichment of primary enrichment broths increase *Listeria* detection. Journal of Food Protection. 62:532-535.

Ray, B. 1979. Methods to detect stressed microorganisms. *Journal of Food Protection*. 42(4):346-355.

Rapid' *L. mono* medium. Retrieved from http://www.bio-rad.com/en-us/product/rapidl-mono-medium?ID=35bad7d6-36ac-4044-b859-d5febbda101a.

Read, R.B. 1979. Detection of Stressed Microorganisms – Implications for Regulatory Monitoring. *Journal of Food Protection*. 42:368–369.

Ritz, M., J.L. Tholozan, M. Federighi, and M.F. Pilet. 2001. Morphological and physiological characterization of *Listeria monocytogenes* subjected to high hydrostatic pressure. Applied and Environmental Microbiology. 67:2240–2247.

Ritz, M., J. Tholozan, M. Federighi, and M. Pilet. 2002. Physiological damages of *Listeria monocytogenes* treated by high hydrostatic pressure. International Journal of Food Microbiology. 79:47–53.

Roso, L 1995. Modeling and predictive microbiology; Development of a new tool for the food industry. Ph.D. Thesis, University Claude Bernard, Lyon, France.

Ryser, E. T., and E. H. Marth. 1999. Listeria, listeriosis, and food safety, 2nd ed. Marcel Dekker, Inc., New York.

Sanaa, M.,B. Poutrel, J. Menard, and F. Serieys. 1993. Risk factors associated with contamination of Raw Milk by *Listeria monocytogenes* in Dairy Farms. Journal of Dairy Science. 76:2891-2898.

Sauders, B.D., K. Mangione, C. Vincent, J. Schermerhorn, C.M. Farchione, N.B. Dumas, D. Bopp, L. Kornstein, E.D. Fortes, K. Windham, and M. Wiedmann. 2004. Distribution of Listeria monocytogenes Molecular Subtypes among Human and Food Isolates from New York State Shows Persistence of Human Disease–Associated Listeria monocytogenes Strains in Retail Environments. *Journal of Food Protection*. 67:1417–1428.

Sauders, B.D., M.D. Sanchez, D.H. Rice, J. Corby, S. Stich, E.D. Fortes, S.E. Roof, and M. Wiedmann. 2009. Prevalence and Molecular Diversity of Listeria monocytogenes in Retail Establishments. *Journal of Food Protection*. 72:2337–2349.

Scallan, E., R.M. Hoekstra, F.J. Angulo, R.V. Tauxe, M. Widdowson, S.L. Roy, J.L. Jones, and P.M. Griffin. 2011. Emerging Infectious Diseases. 17: 7-15.

Silliker, J.H. 1986. *Listeria monocytogenes*. Food Tech. 40(8), 24.

Speck, M. L. and Ray, B. 1977. Effects of freezing and storage on microorganisms in frozen foods: a review. *Journal of Food Protectection*. 40, 333-336.

Tharrington, G., and K.M. Sorrells. 1992. Inhibition of *Listeria monocytogenes* by milk culture filtrates from *Lactobacillus delbrueckii* subsp. *lactis. Journal of Food protection. 55:542-544.*

The control and management of listeria monocytogenes contamination of food. (2005). Dublin: Food safety Authority of Ireland, FSAI).

Thouvenot, P., G. Vales, H. Bracq-Dieye, N. Tessaud-Rita, M.M. Maury, A. Moura, M. Lecuit, and A. Leclercq. 2018. MALDI-TOF mass spectrometry-

based identification of Listeria species in surveillance: A prospective study. *Journal of Microbiological Methods*. 144:29–32.

Vlaemynck, G.M., and R. Moermans. 1996. Comparison of EB and Fraser enrichment broths for the detection of *Listeria* spp. and *Listeria monocytogenes* in raw milk dairy products and environmental samples. Journal of Food Protection. 59:1172-1175.

Walker, S., P. Archer, and J. Banks. 1990. Growth of Listeria monocytogenes at refrigeration temperatures. *Journal of Applied Bacteriology*. 68:157–162.

Warburton, D. W., J. M. Farber, A. Armstrong, R. Calderia, T. Hunt, S. Messier, R. Plante, N. P. Tiwari, and J. Vinet. 1991. A comparison study of the ‘‘FDA’’ and the ‘‘USDA’’ methods for the detection of *Listeria monocytogenes* in foods. International Journal of Food Microbiology. 13:105–117.

Wehr, H. M., and J. F. Frank. 2004. Standard methods for the examination of dairy products (17th ed.). American Public Health Association, Washington, DC.