UNDERSTANDING THE LIMIT: A REVERSE QUANTITATIVE MICROBIAL RISK ASSESSMENT TO INVESTIGATE LOW-LEVEL CONCENTRATIONS OF LISTERIA MONOCYTOGENES IN APPLE PACKINGHOUSES

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ABSTRACT

Listeriosis accounts for 27.6% of all foodborne disease deaths and has a hospitalization rate of nearly 95%, despite the United States' adoption of a "zero tolerance policy" for *L. monocytogenes* levels on foods. Other countries have adopted a quantitative threshold of the pathogen on Ready-to-Eat Commodities, while the US typically integrated Environmental Monitoring Programs to monitor the environment for indicators of the pathogen itself. This allows for sanitation efforts to be directed in presence/absence testing for indicators of *L. monocytogenes, Listeria spp.*; however, these monitoring procedures for produce do not quantify levels of *L. monocytogenes* contamination. Instead, surfaces are tested for the presence of indicators for the sanitation of the production environments. This study uses Quantitative Microbial Risk Assessment (QMRA) to analyze a Listeriosis outbreak from 2014 associated with contaminated caramel apples to investigate the potential presence of low-level concentrations. QMRA is a modeling framework that commonly uses environmental sampling data and mathematical modeling of fate and transport dynamics to characterize the likelihood of adverse health effects due to pathogen exposure. However, in reverse, epidemiological study results from outbreaks can be used to estimate pathogen concentrations in environmental matrices. Thus, a reverse QMRA (rQMRA) was developed to determine the concentration of *L. monocytogenes* present within apple packinghouses associated with the 2014 caramel apple outbreak to better understand the limitations of current detection methods for pathogens, such as *L. monocytogenes*. To capture human variability, two models were constructed with four consumer handling scenarios based on storage temperature (7 °C, twenty-five °C) and storage duration (1 day, 1 week). For immunocompetent populations, the mean estimations were between 0.25 and 0.34 (CFU/g) for the various storage conditions. Much lower median concentrations (0.02-0.07 CFU/g) were estimated for the pregnancy - stillbirth endpoint model. This study indicates that the potential non-zero concentration of pathogens in produce houses calls on the need to integrate prevalence data with more quantitative data to better investigate the potential of missed sampling zones, detection limitations in food safety technology, and to provide more evidence-based reasoning to support policy reform in the future.

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Chapter 1: Background and Motivation

1.1 Michigan Apples

The United States' apple industry is valued at over 3 billion dollars for the 2022-2023 Fiscal Year producing nearly 11 billion pounds of apples [USDA, National Agricultural Statistics Service 2017-2022]. Michigan is a leading contributor to the nation's apple production, contributing 10.2% of all apples produced and projected to increase production in the next few years by 68%. With 775 family-farms in Michigan supporting agricultural production, financial decisions for farm management are critical especially in mitigation of hazards of concern (Michigan Apples, 2022). Impacts such as pathogenic outbreaks do not affect just the production line contaminated but the entire industry as the trust between consumer and product are impacted (Manning, L., & Monaghan, J., 2019). Knowing this, it becomes critical to better support Michigan apples growers in their decision making by learning from these outbreaks in hopes of preventing future outbreaks and impacts to the apple industry.

1.2 2014 Caramel Apple Listeriosis Outbreak

The Center for Disease Control and Prevention put a national eye on the apple industry as they worked with local health departments to better understand the Listeriosis outbreak in 2014 from individuals consuming contaminated caramel apples. The outbreak was investigated by the Center for Disease Control & Prevent (CDC), in which they discovered the source of contamination coming from an apple packing company in California (CA). The hazard of concern was identified as *Listeria monocytogenes* in which 35 individuals reported to have consumed the recalled apples. Of the 35, 34 were hospitalized and the outbreak resulted in 7 deaths. 11 Pregnant women were also exposed within the 35 individuals associated with the outbreak, that resulted in 1 stillbirth. 12 states as far as North Carolina were impacted in this deadly outbreak and becoming the focus of apple packinghouses across the country, including Michigan, to learn from this outbreak to continue to refine and improve mitigation strategies for pathogens such as *Listeria monocytogenes* (Angelo, KM, 2017). To support mitigation strategies for apple growers, policies and guidelines were implemented to minimize these outbreaks; despite these policies and support, outbreaks on fresh produce, such as apples, continues to be the center of concern for stakeholders involved requiring for

an analysis into those policies and guidelines for monitoring and controlling *L. monocytogenes* in fresh produce handling environments.

1.3 Policy & Guidelines for Monitoring and Mitigating *Listeria monocytogenes* in produce environments

Listeriosis outbreaks have been a primary focus for public health officials for decades, especially with more refined knowledge of its ability to be transmitted through food (Ciesielski, C. A., et al., 1988; Schlech III, W. F., et al., 1983; Kozak, J., et al., 1996; Beuchat, L.R., 1996). As a response, the United States adopted a "zero tolerance" policy for *Listeria* on Ready-to-Eat (RTE) food commodities (Ciesielski, C. A., et al., 1988). Other global agricultural leaders took other approaches in their nation's method of quantifying an acceptable threshold of *Listeria* on items such as fresh produce. Canada and the European Food Safety have adopted similar thresholds in which 100 Colony Forming Unit (CFU) & 10-100 CFU per 25-gram (g) sample has been established, respectively (Luher, P., et al., 2011; Bergis, H., et al., 2021; Csadek, I., et al., 2011). More recently, in 2011, a new piece of legislature has been established that enables the Federal Drug Administration (FDA) to focus on preventing food safety problems more proactively (USDA, 2019). The United States' approach looks towards understanding the food handling environment and mitigating the pathogen during manufacturing. This aligns with the emphasis of food safety national programs which work to incorporate preventative measures that identify critical control points of concern through a Hazard Analysis of Critical Control Points (HACCP) to support cleaning and sanitizing efforts, which changed in an approach from reactive to proactive with the introduction of the Food Safety Modernization Act (Shank, F.R., et al., 1996; USDA, 2015). The Food Safety Modernization Act (FSMA) has been considered one of the most progressive food safety reforms to occur within the past 70 years (Szymczak, B., et al., 2020; Grover, A. K., et al., 2016; Boys, K.A., et al., 2015). In it, contains numerous new standards on fresh produce handling (USDA, 2015). One critical new added measure described within the act is the integration of an Environmental Monitoring Program with an enhanced and updated HACCP to better identify mitigation strategies for hazards of concern, in this context *Listeria spp.*

1.4 Environmental monitoring programs

In the food and beverage industry, environmental monitoring programs are utilized for variety of hazards of concern, for the context of the Listeriosis outbreaks food manufacturers often develop pathogen environmental monitoring program for direct monitoring of microorganisms, such as *Listeria* (Simmons, C.K., et al, 2018; Grocery Manufacturer's Association, 2014; United States Food and Drug Administration, 2017; Innovation Center for U.S. Dairy, 2015; United Fresh Produce Association, 2013; United States Department of Agriculture Food Safety and Inspection Service, 2014). A Pathogenic Environmental Monitoring Program (PEMP) is a defined program for monitoring the environment of a food manufacturing facility for pathogens of concern (USDA 2015; International Organization for Standardization, 2015; USDA. 2018; Wiedmann, M., et al., 2020). The goal of these programs is to find and eliminate pathogen contamination in processing environments; the program's purposes are two-fold by verifying an overall food safety systems & to provide early indication of potential food safety hazards. These PEMPs allow for food & beverage manufacturers to have the ability to ensure productivity gain in completing customer orders, reduction in costs for recalls, and to mitigation risk of adverse health outcomes for purchasers of the commodities (USDA, 2017 & Wiedmann, M., et al., 2020).

Food safety in these food handling environments is not a new concept. Classical food safety techniques have been applied for decades centered around quality systems guided by Hazards Analysis of Critical Control Points (HACCP) (USDA, 2015). HACCP is a preventative food safety strategy that applies a systematic approach in identifying and assessing the risks of potential hazards from food and potential mitigation strategies at those critical contamination points (USDA, 2015 & Wiedmann, M., et al., 2020). Pre-requisites are required for the HACCP plan to include items such as documents to verify pest control, Standard Operating Procedures (SOP), and Good Manufacturing Practices (GMP) that prove a holistic plan for ensuring contamination points are identified, standardized, and with controls for mitigation at those critical control points USDA 2015; International Organization for Standardization, 2015; USDA. 2018; USDA, 2017). While opportunities exist for manufacturers to test their commodities directly, it becomes challenging to know to what extent and how many apples to sample and if that is representative of the

potential for contamination (Global Food Safety Initiative, 2018; Jespersen, L., 2017, Wiedmann, M., et al., 2020). Instead, EMPs and PEMPs allow for consistent testing of the entirety of the food safety environment to be more indicative of not just the commodity but the entire food handling process's cleanliness from contamination (USDA 2015; International Organization for Standardization, 2015; USDA. 2018; USDA, 2017; (Simmons, C.K., et al, 2018; Grocery Manufacturer's Association, 2014; United States Food and Drug Administration, 2017; Innovation Center for U.S. Dairy, 2015; United Fresh Produce Association, 2013; United States Department of Agriculture Food Safety and Inspection Service, 2014). These monitoring programs are not new but were made required in 2011 by the Food Modernization Safety Act that shifted food manufacturers, such as apple packinghouses, to be required to show documentation to verify preventive control in their EMP plans, which include PEMPs (Boys, K.A, et al., 2015 & Wiedmann, M., et al., 2020). Now with it being required, numerous guidelines and resources were developed by governmental regulating bodies to guide industry leaders in all the aspects of EMPs & PEMPs to support implementation (20-27). For PEMPs associated with monitoring *Listeria* has identified *Listeria spp.* to be an adequate sampling target as an indicator for *Listeria monocytogenes*. By using the indicator as opposed to the pathogen, testing results for the general species generally provides faster results to identify those areas of concern and implement a cleaning and sanitization regiment. There are only two circumstances in which *L. monocytogenes* is required to be directly tested for: (i) if a recurring detection occurs in any zone after corrective action and (ii) *Listeria spp.* detection in zone 1. HACCP plans for *Listeria spp. / L. monocytogenes* are curated by each food manufacturers and in conjunction with PEMPs allow for the entire process to be considered in how we mitigate at critical control points and how we sample the environment for potential contamination (USDA. 2018; USDA, 2017; (Simmons, C.K., et al, 2018; Grocery Manufacturer's Association, 2014; United States Food and Drug Administration, 2017; Innovation Center for U.S. Dairy, 2015; United Fresh Produce Association, 2013; United States Department of Agriculture Food Safety and Inspection Service, 2014; Wiedmann, et al., 2020).

When constructing PEMPs, numerous factors are carefully considered before implementation including the selection of sampling sites, the frequency of sampling, and the equipment needed to carry out

the monitoring (Wiedmann, M., et al., 2020). Constructing the sampling sites begins with the stage of the process and identifying steps prior to pathogen mitigation stages. Once identified considering the proximity to food is considered in which the Zone Method is applied as seen below in Figure 1.

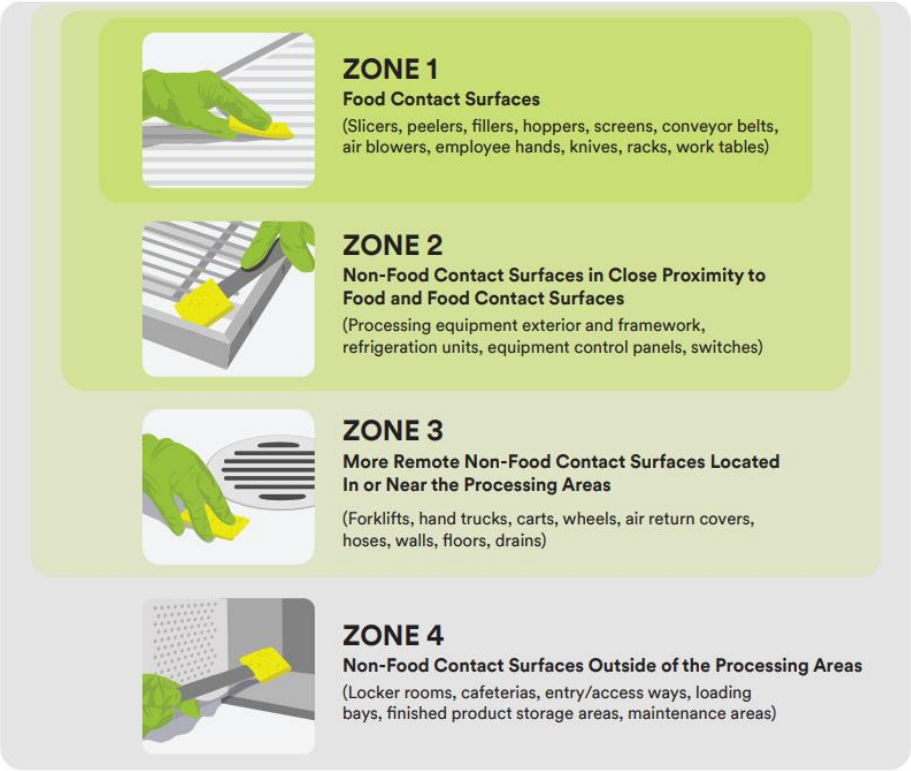

Figure 1: Zone Method used for planning environmental monitoring programs including the sponges as sampling tools for fomite surfaces in each zone (1-4) (Wiedmann, M., et al., 2020)

The zones are coding numerically in which the lower the value indicates a closer proximity to the food commodity of interest. Zone 1 being direct contact equipment (conveyers, rollers, peelers, etc.), followed by Zone 2 being near contact surfaces (sides of process equipment, etc.). Zone 3 contains items considered distant from the food commodity but in the processing room (Forklifts, phones, drains, etc.). Zone 4 is the final zone that contains items outside the Ready-to-Eat (RTE) room (locker rooms, loading docks, etc.). Once the processing of equipment of concern has been identified, then it became imperative to investigate each piece of equipment of interest and identify spaces that may allow buildup to support bacterial survival, with priority in identifying the most difficult places to clean as those are the areas in

which the most sampling should occur. . Figure 2 below, highlights some of the potential harborage sites for bacterial survival.

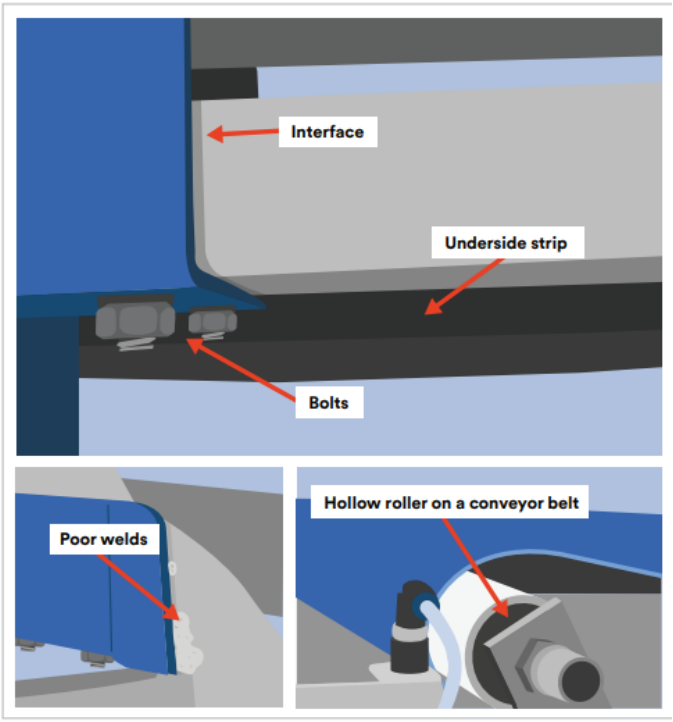

Figure 2: Potential harborage sites for *Listeria spp.* on food handling equipment relating to apple packinghouses such as bolts, welds, hollow rollers, and underside stripping of conveyors that are challenging to clean allowing bacterial build-up (Wiedmann, M., et al., 2020)

The sites for sampling often indicate the frequency at which to sample with recommendations to sample Zone 1 daily and Zone(s) 2-4 weekly. When sampling, detection assay kits such as the 3M™ Molecular Detection Assay 2 – Listeria kit are utilized in conjunction with sponges or swabs. The sponges/swabs are placed on fomite surfaces identified and then sampled in a crisscross pattern utilizing aseptic techniques in the field. Once sampled, the sampling tools are placed into broth for detection of Listeria spp. as captured below in Figure 3.

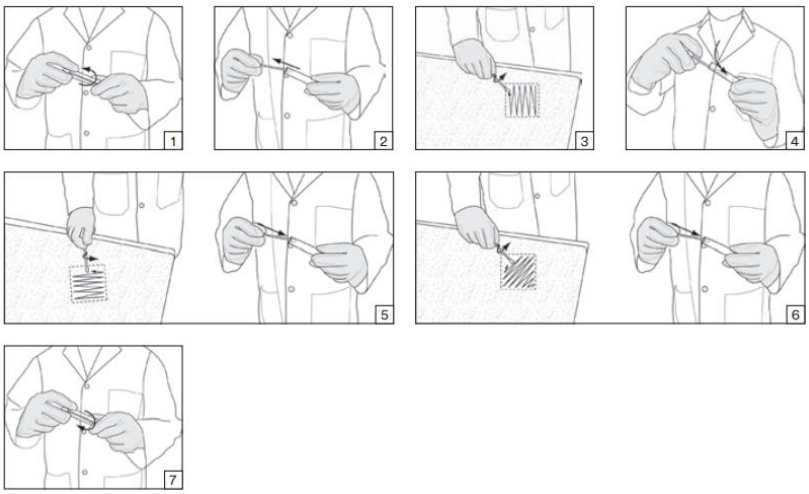

Figure 3: Aseptic Technique of sampling for *Listeria spp.* utilizing sponge-sticks/swabs in conjunction with detection assay kits by sampling fomite surfaces in a crisscross pattern prior to assay analysis (Wiedmann, M., et al., 2020)

Despite guidance and regulations around food manufacturers of RTE foods such as apple packers on the ways to implement PEMPs for *Listeria* there are outbreaks (2014 Caramel Apple Listeriosis Outbreak). Within these monitoring programs, there still are limitations in the current state of environmental monitoring. One potential limitation is the concept of missed sampling which occurs when sampling a zone and receiving an Absence (-) result in which *Listeria spp.* are present. One such mechanism to allow this could be the presence of low-level concentration levels of the pathogen that are below the limit of detection. Thus, to better mitigate *Listeria monocytogenes* from these apple packinghouses, the storage and handling processes must be analyzed to investigate this potential mechanism for missed sampling events.

1.5 Research Questions

To better understand *L. monocytogenes* in the storage and handling processes, this study aims to develop a reverse Quantitative Microbial Risk Assessment to connect published epidemiological data to pathogenic concentration levels in apple packinghouses. This study looks towards answering the following research questions:

1. Are there more critical control points in the pathway than what has been identified?

2. Are the current interventions health protective?
3. How are environmental monitoring results interpreted?
4. How do we better mitigate the risk of exposure to *L. monocytogenes* in food safety environments?

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Chapter 2: Planning, Scoping, & Problem Formulation

2.1 Introduction & Framework

The risk assessment required a framework to properly scope, conceptualize, develop, and implement the assessment with a constant dialogue with the public stakeholders and community leaders to support when the literature available was not enough. The Environmental Protection Agency (EPA) developed the Cumulative Risk Framework to inform and evaluate multiple agents and stressors from the environment to identify associated risk of adverse human health effects from exposure (EPA, 2003). In context, the hazard of concern from the 2014 Caramel Apple Outbreak was identified as *L. monocytogenes* allowing us to leverage the framework to analyze the risk of exposure as this pathogen being the independent pathogen (Angelo, K.M., et al., 2017). Typically, in environmental monitoring *Listeria spp.* are monitored in practice as it is generally slower to obtain specific results on *L. monocytogenes* (Wiedmann, M., et al., 2020). For this risk assessment, *Listeria monocytogenes* will be used as the primary pathogen of concern with an understanding that in practice *Listeria spp.* is analyzed in monitoring for *L. monocytogenes*. Figure 4 below allows the framework to be visualized looking at how the critical components (Planning/Scoping/Problem Formulation, Risk Assessment, and Community Involvement) interact with one another to ultimate guide and inform decision making.

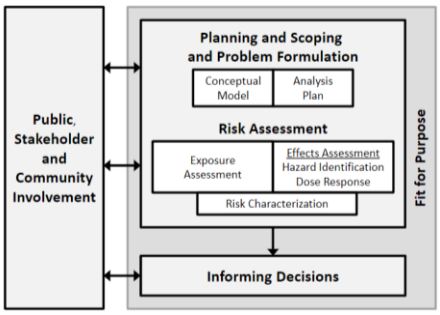

Figure 4: Environmental Protection Agency's flowchart for risk assessment planning and scoping with problem formulation (EPA, 2003)

2.2 Methods

This framework allows for multiple sources of data synthesis to be leveraged in conjunction with the literature to better inform the risk model. Four primary sources were utilized to inform decision making including: (i) Interviews with stakeholders & industry leaders, (ii) site visit(s), (iii) Conceptual model, and (iv) Literature Review.

In February 2022, apple packing sites was visited in Michigan with a small group of collaborators on the project to look at apple packinghouses from the inside. We were able to visit two separate apple packinghouses in which we walked through the process system stage by stage to better understand the context of the risk assessment. This visit provided primary contacts that were able to support informing the risk model. In August 2022, the team went to a clinic sponsored by the Michigan Tree Fruit Commission looking to create a formal space for apple packers and researchers to come together and learn about research findings and implications for their packinghouses. Primarily, this clinic was centered around Controlled Atmosphere (CA) Storage. Numerous leaders, including the primary contacts, attended and provided more opportunities to create dialogue with public stakeholders, specifically around this storage system. These visits in conjunction with meetings and conversations with leaders in the apple industry in Michigan were used to ensure the Public Stakeholders and Community Involvement were accomplished as part of the framework. To support the Planning, Scoping, and Problem Formulation and the Risk Assessment the conceptual model and literature review were the primary sources. With support from apple leaders, a conceptual model was able to be constructed a conceptual model investigating the farm to fork pathway from storage to consumption as seen below in Figure 5.

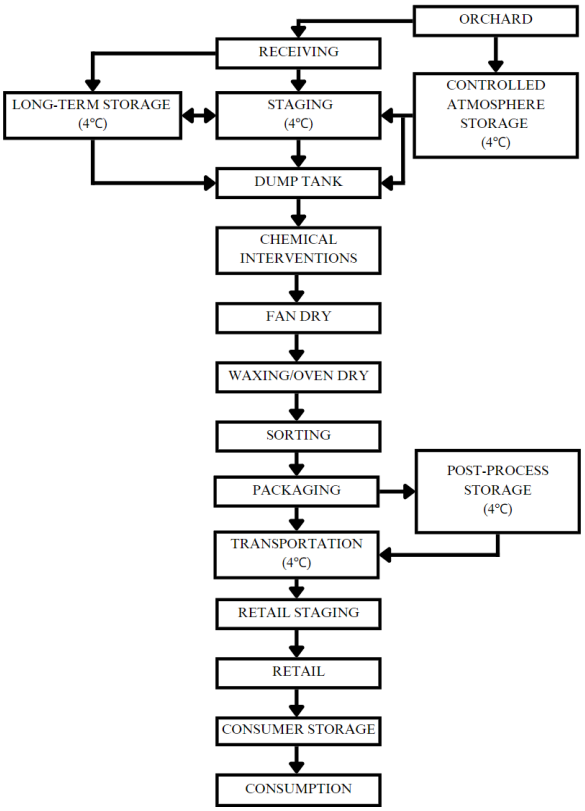

Figure 5: Conceptual Model depicting the compartmental model of the apple processing stages, transportation, retail storage, consumer storage, and ultimately consumption

With the conceptual model developed, the Literature Review was used to synthesize data and parameterize the conceptual model to develop a mathematical model to be integrated into the risk assessment. To better guide the parameterization of the model, an analysis plan was constructed investigating modern mathematical frameworks and methodology that could be used to inform on the risk of human health outcomes from pathogenic exposure.

2.3 Analysis Plan

The analysis plan adopted was grounded in the Quantitative Microbial Risk Assessment paradigm. Quantitative Microbial Risk Assessment (QMRA) is a modelling approach that incorporates microbial fate & transport, exposure factors, and dose response relationships to calculate the risk of adverse human health

effects from exposure to pathogens (Haas, C.N., et al., 2014). QMRA can be used to support evidence-based policy making for the management of infectious outbreaks by quantifying human health risks and the impact of mitigation choices with probabilistic modeling. The framework consists of distinct components that include Hazard Identification, Exposure Assessment, Dose-Response, and Risk Characterization (Soller et al., 2010; Haas et al., 1999; Hunter et al., 2003, Hajare, R., et al., 2021a; Hajare, R., et al., 2021b).

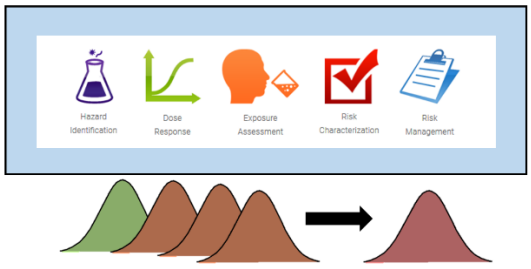

Figure 6: Quantitative Microbial Risk Assessment Framework of Hazard ID, Exposure Assessment, Dose-Response, and Risk Characterization that help to inform Risk Management decisions

Figure 6 depicts the distinct stages of the QMRA framework and how they work together to inform risk management. Hazard identification refers to the identification of pathogen specific characteristic to better understand the microbe of concern within the model; This step involves information gathering for the microbe independent of the environment to understand aspects such as microbial hazards, transmission routes, sensitive populations, health outcomes, and morbidity and mortality rates. Once the target has been identified, the exposure assessment involves describing the pathway from the source of the pathogen in the environment to the point of exposure. Parameters needed within an exposure assessment often include pathogen prevalence in the environmental matrix, pathogen concentrations, intervention efficacies, microbial growth conditions, inactivation rates, and transfer rates. The output of an exposure assessment is a simulated distribution of the exposure dose- the number of pathogens an individual contacts, ingests, or inhales. Once the dose has been calculated, it is inputted into a dose response model. Haas et al (2014) identifies the dose-response model's objective as the characterization of a mathematical relationship

between the exposure dose and the likelihood of occurrence of an adverse consequence, such as infection, illness, or death. Risk characterization is the incorporation of the exposure assessment and dose response assessment using Monte Carlo simulations. Monte Carlo simulations can be run within programs that have been utilized for uncertainty sensitivity analysis to better understand the weight each parameter has to assist in the refinement and validation of the risk assessment.

To fully inform the risk assessment numerous categories needed to be investigated and then leveraged within the modeling of potential low-level concentration of *L. monocytogenes*. To accomplish the risk assessment the four sources for data collection were used to inform the Planning, Scoping, and Problem Formulation portion of the framework.

2.4 Results

The findings of the initial stage of the Cumulative Risk Framework (Planning, Scoping, and Problem Formulation) were able to support informing each compartment of the QMRA framework investigating: (i) Hazard Identification to better understand the pathogen, (ii) Dose-Response to understand the relationship between exposure to the pathogen and adverse health outcomes, (iii) Exposure Assessment to develop a mechanistic model of the pathogen's behavior in the environment, and (iv) Risk Characterization by utilizing Monte Carlo Methods with parameters from Dose-Response and Exposure Assessment to better understand variability and uncertainty in the assessment.

2.4.1 Hazard Identification

Listeria monocytogenes is a well-known gram-positive bacterium known to be a mechanistic agent of listeriosis, an infection often from contaminated whole food commodities and known to have high morbidity and mortality for those infected (Fratamico et al., 2005; Hew, C.M., et al., 2006). First discovered in humans by J. Dumont in 1918 from a meningitis case and later proven in the 1980s to be an emerging pathogen of concern (Shlech, W. F., 1983), *L. monocytogenes* continues to demonstrate symptoms of concern after infection such as septicemia and infections of the central nervous system especially within groups such as those immunocompromised, the elderly (above 65 years), and pregnant women (Charlier, et al., 2012, 2017; Chersich, M.F. et al., 2012). *L. monocytogenes* (Lm) has several determinants, the most

studied of which include the surface proteins internalin A (InlA) and internalin B (InlB), which have been identified as requirements for infection through binding on the host cell receptor. Despite there be adequate research around several key surface proteins promoting the ability to adhere and infect host cells, there are numerous determinants of *L. monocytogenes* that have been identified suggestion a higher-order of complexity for the cell-adherence process (Kathariou, S. et al., 2002; Cossart, P. et al., 1998; Dramsi et al., 2000; Kuhn et al., 1999). Intragastric infections from oral consumption are among the most common allowing for the development of several animal models identifying the virulence capacity across strains; however, notably a substantial portion of studies around *L. monocytogenes* intragastric infections utilize mice, who lack the fundamental receptor protein (E-cadherin) ultimately hiding a limitation within the current routine studies around virulence specifically with oral infections of *L. monocytogenes*.

Adhesion to fomite surfaces is also possible as a function of the Listeriolysin O-region. The Listeriolysin O region is a cluster of genes in the bacterium that include genes for factors such as protein (ActA), two phospholipases, and the hemolysin (Listeriolysin O), which work in conjunction to support the intracellular mobility of the pathogen upon infection. Once within the host cell, the pathogen is emerged within a vacuole that triggers the expression of Listeriolysin O to promote the escape of the pathogen into the cytoplasm creating a cycle within the cytoplasm as the ideal environment for pathogenic survival and replication (Kathariou, S., 2002).

Biofilm has been identified as a community of microorganisms adhering to a surface, often in food processes both biotic and abiotic surfaces (Costerton et al., 1995). The production of which contains three critical steps: adhesion, maturation, and disbursement. There are large data gaps in areas such as modelling biofilm formation, understanding the dimensionality order of structure, and variation within genetic lineage (Renier et al., 2011; Rieu et al., 2008; Doijad et al., 2015). Not only is the formation of the biofilm unique, but the strength of attachment varies with categories of weak, moderate, and strong existing to classify the ability to better understand the firm attachment to abiotic surfaces (Da Silva and De Martinis, 2013; Reis-Teixeira et al., 2017; Harvey et al., 2007). Studies have been conducted to identify unique qualities *L. monoctyogenes* biofilm contains through numerous methods, the most popular being Scanning Electron

Microscopy (SEM) (Pringle, F.M., et al., 2001; Chavant et al., 2002; Borucki et al., 2003). More recent developments have identified the pathogens' ability to synthesize biofilm in challenging environments, such as hydrophilic surfaces (Chavant et al., 2002). Understanding the formation and persistence is nuanced; researchers have been able to identify molecular determinants for the formation of Listeria biofilm to such as aspects as signal transduction systems, surface structures, and information pathways. *L. monoctyogenes* ability to create a cellular barrier for persistence in challenging environments is supplemented by its ability to survive in extreme conditions before biofilm formation.

Studies have been conducted to elucidate why *L. monocytogenes* is emerging within the food industry. *L. monocytogenes* is unique in its ability to persist in unideal environments such as being able to survive in the range of -0.4 to 50 ℃ and in both acidic and basic environments within the pH range of 4.3 to 9.4 (Kathariou, S., 2002). The pathogen's ability to tolerate acidic environments, such as the stomach, highlights concern for the human health effects upon ingestion. The degree of tolerance is often pre-determined by the current growth stage, but numerous studies have shown how the acid-tolerant mutants increase virulence and is an additive cause of concern within the pathogenicity of *L. monocytogenes* (Cotter, P.D., et al., 1999; Marron, L., et al., 1997.; Gahan, C.G., et al., 1996).

The mechanistic process of pathogenic survival encompasses how the pathogen moves throughout the system (transport), the form and characteristics it shapes by the end of the process (Fate) and the ability of the pathogen to survive through the fate and transport (persistence) (Jung, S., et al., 2019a; 2019b). With improved knowledge of the complex mechanisms of survival for the pathogen within the food process system, interventions through cleaning and sanitization procedures have been implemented to ensure daily intervention within facilities (Jessen, B., et al., 2003; Rotariou, O., et al., 2014). Nevertheless, error in the cleaning and sanitation process has been reported within numerous facilities and has been identified to encourages higher retention of the microbe on food surfaces to ultimately increase the likelihood of contamination of fresh, whole apples (Autio, T., et al., 2003; Ferreira, V., et al., 2014; Lundén, J.M., et al., 2003a; Lundén, J.M., et al., 2003b; Møretrø, T., et al., 2004; Tompkin, R.B., 2002; Wulff, G., et al., 2006).

Scientists have investigated this pathogen and its ability to survive within these packinghouses typically by utilizing environmental monitoring to support informing prevalence on food contact surfaces using presence/absence testing of the indicator organisms (*Listeria spp.)* using rapid techniques with results as quick as 48 hours after sampling (Ruiz-Llacsahuanga, B., et al., 2021a). Studies have also investigated the species as well as the pathogen of concern investigating the prevalence of both indicator and pathogen on apple packing processing surfaces utilizing the Bacteriology Analytical Manual as the guiding method for detection of *Listeria spp.* and *Listeria monocytogenes* (Estrada, E.M., 2020 & Ruiz-Llacsahuanga, B., et al., 2021b, Hitchin, A., 2017). Understanding the prevalence of the pathogen can be used to know high-level areas within the PEMPs, typically to identify areas that require more frequent cleaning and sanitation efforts.

Once contamination has occurred, eradication and removal becomes especially challenging due to the pathogen's known biofilm formation; additively, Listeria's ability attach to numerous surface types contributes to an elevated challenge in eradicating the pathogen and increasing the likelihood of cross-contamination. The persistent cells on food-surfaces are often sources of outbreaks and highlights the need to better understand how the pathogen survives within the storage and handling practices to better understand the intervention needed at critical points to minimize the probability of colonization (Lundén, J.M., et al., 2002; Reij, M.W., 2004).

2.4.2 Exposure Assessment & Apple Packinghouses

Once an understanding of the hazard of concern and monitoring opportunities of indicators has been solidified, it becomes essential to understand the amount exposed to individuals associated with the 2014 Listeriosis outbreak during consumption of the caramel apple. There are a variety of packinghouse designs, process organizations, and interventions for microbial reductions used throughout the Michigan apple industry. These facilities have high traffic areas that allow cross-contamination from the field. Sites such as flooring, storage equipment, cooling units, walkways, and drains have all been identified as harborage areas for Listeria within packinghouses (Wiedmann, M., et al., 2020). Figure 7 contains the conceptual model as seen below.

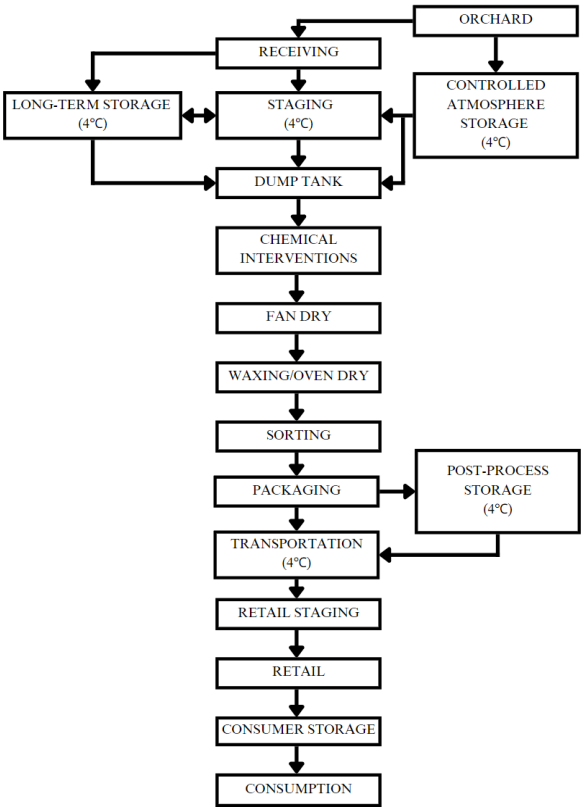

Figure 7: Conceptual Model depicting the compartmental model of the apple processing stages, transportation, retail storage, consumer storage, and ultimately consumption

Both literature and expertise from leaders in industry leaders were used to construct the conceptual model (Simonetti, T., et al., 2011; Ward, S., et al., 2022). Apples are taken from the orchard and then either placed in a controlled atmosphere room, prepped for long-term storage, or prepped for processing. More details on the differences in long-term storage and controlled atmosphere storage will be discussed below, but within these stages, apples are stored for up to 9 months before entering the staging to be processed. Once in the processing system, the apples are chemically treated, dried, applied a wax, dried again, then sorted before packaging. Once packaged, the apples are transported to retailers in which they are staged before being placed in retail for purchase. The consumer then can purchase the apple and practice a variety of consumer storage practices before ultimate consumption.

2.4.2.1 Stage Distinctions

The conceptual model provides three distinct stages in the processes as seen below in Figure 8. With Stage 1 (green) showcasing the pretreatment stage, which contain compartments prior to the processing system including the controlled atmosphere room. Stage 2 (blue) shows the initiation of the processing system by placing whole apples into dump tanks that are filled with processing water to support the movement of apples through the process without damaging the food. This stage ends before the packaging step when the first direct human contact occurs in the system. Stage 3 (orange) depicts the apple's experience from the packaging at the apple packinghouse through the consumption event of the contaminated apple.

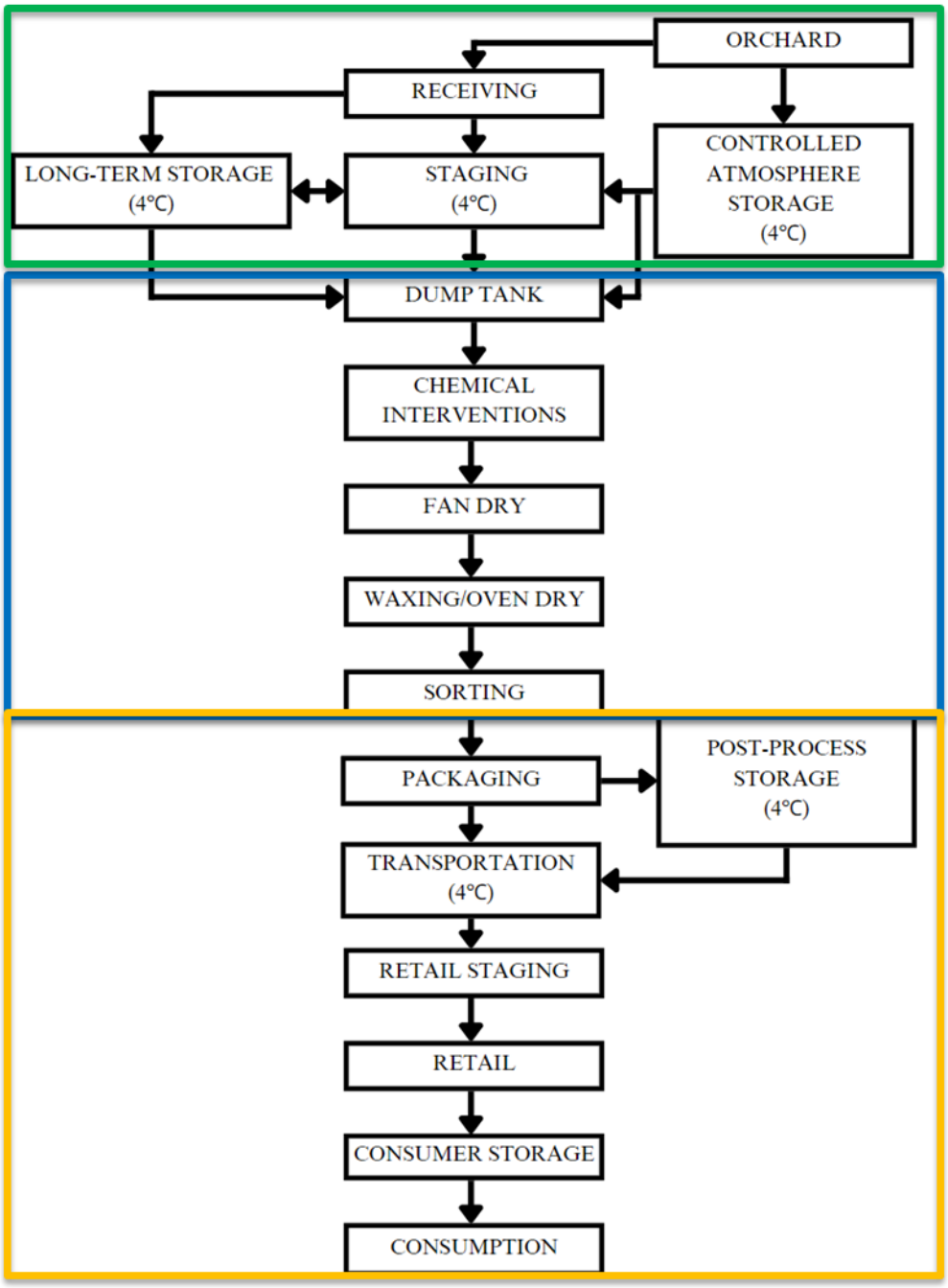

Figure 8: The conceptual model of the farm-to-fork pathway of a Ready-to-Eat (RTE) apple with categorized compartments of the conceptual model into three primary stages including: pretreatment (green), processing (blue), and packing (orange)

2.4.2.2 Pretreatment Stage

From the orchard, apples are transported to the manufacturing facility by forklifts primarily in apple bins. These bins have two primary materials they are made of: wood and plastic. These bins contain the full content of the apple from the orchard in its natural state, meaning items such as organic debris, dirt, leaves, etc. are also found within the apples along with the commodity itself. Once at the facility, apples are either staged and then processed or stored. Long-term storage is one option in which the apples are stored in a room at 4℃ for extended periods of up to 9 months. Similarly, another option is to utilize Controlled Atmosphere (CA) Storage, which is slightly more sophisticated (Sabban-Amin, R., et al., 2011). The CA room is kept between -1°C to 4°C with a high air exchange allowing for higher proportion of nitrogen (N2) than Oxygen and Carbon Dioxide within the room to slow the production of ethylene production that affects the ripening rate of fresh, whole apples. The room is also held at 90 – 95% Relative Humidity to assist in the long-term storage of up to 9 months of CA storage. The room hosts numerous machines to assist in the air exchange, moisture control, and air quality including condensers (Mditshwa, A., et al., 2018). The rooms are often large in volume and allow for large quantities of apples to be stored within storage bins. Though Controlled Atmosphere Rooms are relatively understudied, recent studies have shown that within these Nitrogen dominated environments that are held near 4 °C, *L. monocytogenes* is not able to grow but is able to maintain through the duration of up to 9-month storage (Sheng, L., et al., 2017). Once farmers are ready to open these rooms and access the apples, the rooms are clear, and the apples are transported to the packinghouse facility for the processing stages.

2.4.2.3 Processing Stage

Once the apples are transported out of the controlled atmosphere, the apples then move to the next compartment within the processing stages. Forklifts transport the apples into the staging area, and they are placed into the water conveyor system, fully emerged in the process water to assist in the delicate handling of the apples. Once submerged the apples move through the flume dump tanks typically include chemical interventions such as Peracetic Acid, Free Chlorine, and Food-Grade Soap. For the baseline model, the assumption being the first dump tank is treated with PAA and then recirculated and then the second flume

dump tank would be treated with Chlorine. After each dump tank – lasting approximately 3-4 minutes - the apples are then treated for 1 minute during single-pass flow by PAA and PAA/Soap after each tank respectively. After moving through the chemical treatments, the fresh, whole apples are then heat treated at 80°F for 3 minutes and then follows a waxing stage that. The waxing process lasts for 30-40 seconds and completes the wet processing stage of the process. Within the waxing process, the final stage of polishing often utilizes a set of roller-brushes, which has been identified as a harborage site for *Listeria spp.* due to their challenges in cleaning and sanitizing these brushes (Ruiz-Llacsahuanga, B., et al., 2021a, Ruiz-Llacsahuanga, B., et al., 2021b, Ward, S., et al., 2022). Once the waxing is complete, the apples are then sorted based on weight, color, and cultivar either manually or automated into the packaging lines transitioning the conceptual model from processing to packaging.

2.4.2.4 Packing Stage

After the processing stage is complete, the workers within the line move towards sorting and packaging the apple. While the direct contact between the apples and workers is shared across the industry at this stage to move the commodities into their transportation vehicle; thus, the risk of infection from ingestion of contaminant is quantified within the model to better understand and implement interventions for worker safety. Upon packaging, the apples move into cold stage and await to be transported to retail and ultimately the consumer. The scientific literature available was not available to inform the timeline of apples during this final stage, especially from transportation to consumption. Public stakeholders were able to inform the project on typical times associated with each stage that can be seen below in Figure 9.

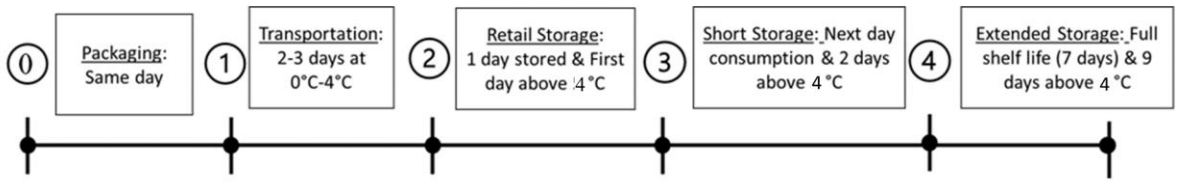

Figure 9: Timeline for an apple within the packaging stage of the conceptual model informed by public stakeholders on duration in each compartment for transportation, retail storage, retail storage, and consumer storage as 2-3 days, 1 day, 1 day, and 7 days respectively

Figure 9 was able to capture the duration of each compartment during this stage of the apple handling. Transportation for the 2014 Caramel Apple Listeriosis Outbreak was sourced from California to states as far as North Carolina taking 2-3 days to complete. During Transportation, the apples are stored under the same conditions as Long-term storage. At retail, the apples are then received, and quality checked before being placed on the shelves, which typically takes a day to accomplish. Once on the shelves, the shelf-life for an apple has been identified as up to 1 week meaning that variability exists in the duration and the temperature stored by apples in which we captured the soonest possible event before consumption (Short-Storage) and the event of full shelf-life occurring before consumption (Extended Storage). Retail storage receiving was identified as the first instance since packaging in which the RTE apple is stored at above 4°C. Making the first day possible for consumption being the second day above 4°C and the full shelf life occurring 9 days above 4°C.

2.4.2.5 Process equipment

Within each of these stages in the farm-to-fork pathway, process equipment of interest can be identified to support the construction of those EMPs/PEMPs in the apple packinghouses. In pretreatment, the forklifts and bins are used in the field and then brought into the pretreatment stage (Simonetti, et al. 2021). These forklifts and bins are suitable vectors for the cross-contamination of the pathogen from the field into the manufacturing site (Long-storage, receiving and/or CA-storage) (Sullivan, G., & Wiedmann, M., 2020). Forklifts, potentially the same from the orchard again, then move the apples from the

pretreatment stage into the processing stage allowing for another point of cross-contamination to occur. During processing, the zone method (Figure 1) and the Hazards Analysis for Critical Control Points can be used to identify the process equipment of interest by stage. Equipment such as dump tanks, conveyor belts, roller-brushes, sorting machines, and workers hands are all within direct contact with the food commodity are confirmed through prevalence studies to be harborage sites for *Listeria spp.* (Ruiz-Llacsahuanga, B., et al., 2021a; Estrada, E.M., 2020 & Ruiz-Llacsahuanga, B., et al., 2021b, Hitchin, A., 2017). Zones 2-4 are also investigated in these studies and similar ones with a primary focus of looking at items such as below process equipment, drains, and the forklifts are analyzed in prevalence studies also (Belias, A., et al., 2020). Packaging contains the first direct contact with workers in which manual packaging from the sorted apples occurs; more often, apple packinghouses are shifting towards automation to mitigate exposure events (Ward, S., et al, 2022). Understanding these stages and equipment of interest is critical in constructing an exposure model and exposure assessment to understand the concentration of the pathogen exposed to individuals during the 2014 outbreak.

2.4.3 Dose-Response Modeling

Once the mechanistic model was informed where the exposed dose can be calculated from the outbreak, the next compartment (Dose-Response) investigates the relationship between the amount exposed to the individual to their probability of adverse human health effects, such as infection, stillbirth, death, etc. In Dose-response modeling, two models have been identified as biologically plausible and used to investigate animal studies looking at the exposure dose to outcomes of interest and can be seen below in Equation (Eq) 1 and 2.

$$P(response) = 1 - \exp(-k \times dose) \quad (1)$$

$$P(response) = 1 - \left[1 + dose \frac{\left(2^{\frac{1}{\alpha}} - 1\right)}{N_{50}}\right]^{-\alpha} \quad (2)$$

In the exponential model, the *k* parameter is the probability of a pathogen surviving and initiating an infection; the beta-Poisson model assumes that there is variation in the probability of a pathogen surviving to initiate an infection, and this variation is represented with a beta distribution. As shown in Eq. 2, α is shape parameter and N50 is the value associated with 50% effect to subjects.

Dose-response models are distinct to three key characteristics: the pathogen of concern, the exposure route, and the endpoint. The pathogen of concern from the 2014 Listeriosis outbreak was identified to be *Listeria monocytogenes* in which several dose-responses have been validated and published within literature. Three primary models exist looking at the typical transmission of *L. monocytogenes* through the oral-fecal pathway typically through consumption of contaminated food commodities with different endpoints of the models being infection, stillbirth, and death. The first model, Golnazarian et al. (1989) compared infectious dose in normal and compromised mice with pathogens (strain F5817) in which the data collected was then used to validate a dose-response looking at infection of the pathogen (Haas, C.N., et al., 1999). A second dose-response model was validated looking at data in which pregnant Rhesus monkeys & Guinea Pigs were pooled and were used to investigate *L. monocytogenes* consumption with the endpoint of stillbirth. Third, a model investigated death as the endpoint, in which mice were used to validate a dose-response model for consumption of *L. monocytogenes* resulting in death. Once the models of best fit are identified, parameter estimation was complete in which those optimized parameters for the models of best fit can be seen below in Table 1.

Table 1: Dose-Response Table containing populations, endpoints, transmission route, and optimized parameter of interest for each model for *Listeria moncytogenes*

Dose- Response Models *Listeria Monocytogenes*

Population	End Point	Host	Route	Best fit Model	Alpha	N50	k
Immunocompetent	Infection	Mice	Oral	Beta - Poisson	0.253	277	-
Immunocompetent	Death	Mice	Oral	Exponential	-	-	1.15E-05
Pregnant Women	Stillbirths	Rhesus Monkey	Oral	Beta - Poisson	0.0422	1.77E+09	-

From Table 1, the best fit dose-response models for infection, death, and stillbirth have been identified as Beta-Poisson (BP), exponential, and Beta-Poisson (BP), respectively. From these models of best fit the alpha, N50 for the BP models and the k-parameter for the exponential model were optimized.

2.5 Conclusion

The findings from the Planning, Scoping, and Problem Formulation components of the framework were critical towards the success of the project. Limitations in the available literature were supported by knowledge from public stakeholders and industry leaders, when possible, to support the development of this data synthesis process. With a refined understanding of the conceptual model and the analysis plan as well as parameterized model, the work created in Chapter 2 was used to inform a reverse Quantitative Microbial Risk Assessment to better understand the concentration of Listeria monocytogenes in apple packinghouses associated with the 2014 Caramel Apple Outbreak (Chapter 3).

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Chapter 3: Risk Assessment

3.1 Introduction

The 2014 Listeriosis multi-state outbreak was associated with *L. monocytogenes* contamination from the puncturing of caramel apples (Glass, K., et al., 2015). Investigations into this outbreak highlighted the pathogen's ability to survive within the caramel apple coating process showcased how *L. monocytogenes* has been identified as an emerging foodborne pathogen of concern (Angelo, K.M., et al., 2017; Salazar, J. K., et al., 2016; Glass, K., et al., 2015; Ward, S., et al., 2022). The prevalence within these packinghouse environments has been acknowledged as a high concern despite interventions existing within the handling process (Tan, X., et al., 2019; Kuttappan, D., et al., 2021; Simonetti, T., et al., 2011). Studies have identified numerous biological properties associated with the transport the pathogen within the farm-to-fork pathway, including biofilm formation, extremophilic properties, and adhesive proteins, however, the understanding of these factors within specifically food processing environments is not well developed as current efforts of genetic subtyping and available data on persistence within engineering controls is limited (Camejo, A., et al., 2011; Coelho, C., et al., 2019; Carpentier, B., et al., 2011).

In the United States, the adoption of a "zero-tolerance" policy for the threshold level of *L. monocytogenes* is practiced utilizing Environmental Monitoring Programs (EMPs) and Pathogen Environmental Monitoring Programs (PEMPs) as a result of the 2011 Food Safety Modernization Act; therefore, environmental sampling is typically conducted utilizing a presence/absence methodology where a positive result indicates need for immediate sanitation of the surface in contact with commodities (Warriner, K., et al., 2009; Center for Food Safety and Applied Nutrition, 2023; Churchill, R. L., et al., 2006). Comparatively, organizations internationally require concentration levels to be below a tolerance threshold such as the countries of the European Food Safety Authority (EFSA) and Canada that have qualified an "acceptable risk level" to be associated with the contamination threshold of 100 Colony Forming Units per gram (CFU/g) of sample (Churchill, R. L., et al., 2006; EURL, 2019; Clarke, J., 2010). Despite a zero-tolerance policy, Listeriosis outbreaks associated with commodities continues to occur in the United States (Buchanan, R. L., et al., 2017; Zhu, Q., et al., 2017). To investigate the efficacy of current

environmental sampling efforts, a quantitative microbial risk assessment (QMRA) was completed herein to estimate the concentrations of *L. monocytogenes* associated with the 2014 caramel apple outbreak. QMRA is a mathematical framework that characterizes the association between exposure events and the risks of adverse human health effects for microbial contaminants (Haas, C.N., et al 2014). The framework is widely accepted to determine risk levels associated with known environmental exposures to contaminated media, such as water quality design criteria, air quality design criteria, and food safety guidelines (Sano, D., et al., 2019; Owens, C. E., et al., 2020; Dean, K., et al., 2020). A forward QMRA typically uses environmental sampling data to estimate human health risks, however this study works to understand and estimate the concentrations of *L. monocytogenes* within the produce packinghouses associated with the 2014 caramel apple outbreak by completing a reverse QMRA. Risk assessors, Michigan apple producers, and food safety policymakers need to know the concentration levels of *L. monocytogenes* associated with outbreaks to better mitigate and minimize contamination events, prioritize intervention strategies, and develop a better understanding of current microbial detection capabilities in relation to the outbreaks. Currently, with the "zero-tolerance" national policy there is little quantitative knowledge about the persistence, transport, and behavior of *L. monocytogenes* in food production and processing environments. The conclusions drawn from the results of this study will help identify gaps in current policies and technologies to better inform intervention selections and handling practices for apple packinghouses and minimize future Listeriosis outbreaks.

3.2 Methods

3.2.1 QMRA Framework

Figure 10: Quantitative Microbial Risk Assessment Paradigm with Compartmental Elements of Hazard Identification, Dose-Response, Exposure Assessment, Dose-Response, & Risk Characterization (Haas, C.N., et al., 2014)

The QMRA Framework, Figure 10, is a compartmental model that allows for discrete stages of the farm to fork pathway to be characterized into: Hazard Identification, Exposure Assessment, Dose-Response, and Risk Characterization. Hazard Identification involves the identification of the pathogen of concern within the risk assessment and the consideration of the biological properties, disease process, and populations of concern. The exposure assessment involves the characterization of the fate and transport of the pathogen through the process being analyzed. For produce packinghouse facilities, an exposure assessment includes the parameterization of the initial contamination event and subsequent chemical interventions, growth dynamics, and handling practices. An extensive review of the literature is often necessary to estimate each parameter, and probability distributions are typically used to capture parameter variability and uncertainty. The outcome of an exposure assessment is an exposure dose: the number of pathogens ingested in an exposure event. The calculated dose when transmitted through its associated route would then be required for the dose-response stage to be completed. The dose-response element of the paradigm correlates the dose ingested to probabilistic outcomes for human health effects. The two biologically feasible microbial dose-response models are the exponential and beta-Poisson models, described in Eq. 1 and 2.

$$P(response) = 1 - \exp(-k \times dose) \quad (1)$$

$$P(response) = 1 - \left[1 + dose \frac{\left(2^{\frac{1}{\alpha}} - 1\right)}{N_{50}}\right]^{-\alpha} \quad (2)$$

In the exponential model, the *k* parameter is the probability of a pathogen surviving and initiating an infection; the beta-Poisson model assumes that there is variation in the probability of a pathogen surviving to initiate an infection, and this variation is represented with a beta distribution. As shown in Eq. 2, α is shape parameter and N50 is the value associated with 50% effect to subjects. Risk Characterization is the final element of the QMRA in which the exposure doses and risks of adverse health outcomes are simulated, and the sensitivity of the risk model to any individual input is investigated. This effort ultimately allows for the concentrations of a pathogen in an environmental matrix to be associated with probabilities of adverse health outcomes. However, in the case of the 2014 outbreak of Listeriosis from caramel apples, the probabilities of adverse health outcomes are known from the epidemiological study that assessed the impacted populations (Angelo, K.M., et al., 2017). The concentration of *L. monocytogenes* on or in the apples is an unknown value. Thus, the QMRA framework can be utilized in reverse (rQMRA) to associate the adverse health outcomes with the concentrations of L. monocytogenes responsible for the outbreak.

3.2.2 Probability of Adverse Health Outcomes

This rQMRA focuses on two populations: 1) immunocompetent adults, and 2) pregnant women. *L. monocytogenes* is associated with a high morbidity rate for pregnant women and may cause stillbirth in their pregnancy. Few risk assessments have been done with this sensitive population however, pregnant women were a population impacted in the 2014 outbreak. This rQMRA aims to estimate the concentration of *L. monocytogenes* on or in apples associated with the outbreak's observed risk of death for healthy adults and the risk of stillbirth for pregnant women. Of the 35 individuals infected, there were 7 deaths. Of the 11 pregnant women infected, 1 stillbirth occurred (Angelo, K.M., et al., 2017).

Several available dose response models for the ingestion of *L. monocytogenes* have been identified with endpoints of stillbirth, death, and infection (Golnazarian, C.A., et al., 1989; Haas, C.N., et al., 1999; Smith, M.A., et al., 2008; Williams, D., et al.,2007). For the pregnant women model who experience stillbirth, this 1/11 statistic will be the endpoint statistic while the immunocompetent adult will be 7/35 representing the 7 deaths within the affected population of 35. At this point, it is critical to acknowledge that more than the 35 individuals connected to the outbreak could have been a part of the impacted population. It was assumed in this study that the 35 individuals connected to the outbreak represented the entirety of the exposed population. This assumption is considered highly conservative as the number of individuals exposed to contaminated apples was likely much higher. The assumption that the 11 pregnant women associated with the outbreak were the only 11 pregnant women exposed, is perhaps less conservative overall, as the population may have been more likely to seek medical attention for minimal or mild symptoms than the average adult. Notably, to minimize variability, the death and stillbirth endpoint dose-response models were selected as the endpoints are considered more certain as clear binary outcomes (occurrence or no occurrence) as opposed to infection endpoints which would appear physically very different for populations.

3.2.3 Dose-Response Modeling

The optimized parameter values for α and N_{50} for the beta-Poisson model with a stillbirth endpoint, and the *k* parameter for the exponential model with a death endpoint are shown in Table 2 (Golnazarian, C.A., et al., 1989; Haas, C.N., et al., 1999). To calculate *dose* with a known probability of response (*P(response)*), the exponential model from Eq. 1 was reconfigured as shown in Eq. 3. Similarly, the beta-Poisson model from Eq. 2 was reconfigured to solve for dose as shown in Eq. 4.

Table 2: Epidemiological Statistics and Parameter Input for the Reverse Quantitative Microbial Risk Assessment Dose-Response Model

Variable	**Value**	**Distribution**
P(death)	0.2	Point Estimation
P(stillbirth)	0.0909	Point Estimation
k, death	1.15 E -5	Optimized Point Estimate
α, stillbirth	0.0422	Optimized Point Estimate
N50, stillbirth	1.78E+9	Optimized Point Estimate

$$dose = \frac{ln(1-P(response))}{-k} \quad (3)$$

$$dose = \frac{\left[(1-P(response))^{-1/\alpha}-1\right]\cdot N_{50}}{(2^{\frac{1}{\alpha}}-1)} \quad (4)$$

3.2.4 Exposure Assessment:

The dose calculated in Eq. 3 and 4 represents the number of pathogens that survived or evaded the current interventions in post-harvest production for apple producers to be ingested by the consumer for either model. The caramel apple scenario is unique in that extensive studies have shown that the surface of fruits often dictates survivability for *L. monocytogenes*: for example, fresh, whole apples cannot provide adequate means to support microbial growth as the surface of apples are known for its acidic surface (pH < 4.0) and low water activity (a_w>0.85) (Angelo, K.M., et al., 2017; Glass, K.A., et al., 2015). The caramelization stage for caramel apples is also expected to eliminate 99% of the pathogens on commodity surfaces; however, when the apple is punctured prior to caramelization, a portion of the stem cavity is mechanically forced inside the apple. The inner core of the stem-cavity provides adequate means for not only microbial survival but growth within the caramel coating (Ward, S., et al., 2022). Thus, to better

understand the concentrations associated with the initial contamination of *L. monocytogenes* within the packinghouses, the exposure assessment must account for the pathogen growth that occurred in the caramel apple, any log-reductions associated with current interventions established by apple producers, and the variability in these values that may be typical of these environments. The farm to fork pathway of fresh, whole apples is captured in the conceptual model; however, to account for caramel apple production with two additional steps: puncturing and caramelization. The exposure model considering these new steps from injection can be seen below in Figure 11.

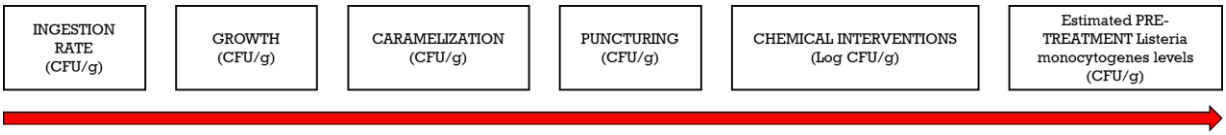

Figure 11**:** Flow diagram of tree fruit packinghouse process for fresh, whole apples undergoing caramelization for RTE caramel apples

Within this exposure pathway, there are two primary points that impact the concentration of *L. monocytogenes* in or on the caramel apple: 1) consumer handling practices that may lead to pathogen growth, and 2) chemical interventions commonly applied in the industry.

3.2.4.1 Growth Scenarios from Consumer Handling Practices:

Packaging of apples occurs the same day, where they are stored in cold storage between 0 °C and 4 °C, which has been identified as a region in which negligible growth occurs for Listeria monocytogenes (Scotter, S.L., et al., 2001). Once the packaging is complete the apples are stored and transported in the same thermal conditions where it takes 1-3 days to deliver the commodities from the source (CA) to the impacted states. Thus, no growth was assumed during the stages that are below the 4 °C mark. However, once the commodity is transported it is then stored at elevated temperatures at retail, typically for a day

before being put on display for inspection in retail storage. The exposure scenario thus assumes there are only 24 hours of time associated with possible pathogen growth in the stem cavity before purchase.

Human behavior results in large variability in the ways that apples are stored, handled, and consumed after purchase; however, two of the most impactful parameters for pathogen growth are time within storage and the storage temperature (Li-Cohen, A., et al., 2002). Knowing this, several scenarios were investigated herein to capture the spectrum of practices expected to be associated with the caramel apple exposure from the outbreak. There are four scenarios investigated within this study that identify combinations of time and temperature practices that are practical for caramel apple handling: refrigerator stored apples consumed the same day of purchase, refrigerator stored apples consumed at the end of shelf-life (7 days), room temperature stored apples consumed the same day of purchase, and room temperature stored apples consumed at the end of shelf-life (USDA, 2015; Sheng, L. et al., 2017). A study conducted by Salazar (2016) evaluated the growth of L. monocytogenes over time at the temperatures of interest for this exposure scenario (5°C/25°C). Using the growth profiles from Salazar (2016), the concentration in the stem cavity before storage and purchase was estimated assuming 2-9 days of growth at 5°C or 25°C, as illustrated in Figure 12.

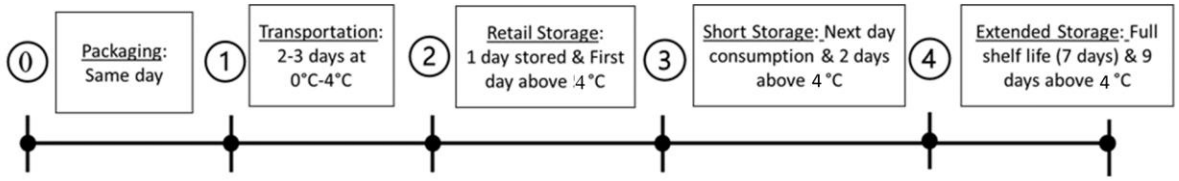

Figure 12: Timeline for an apple within the packaging stage of the conceptual model informed by public stakeholders on duration in each compartment for transportation, retail storage, retail storage, and consumer storage as 2-3 days, 1 day, 1 day, and 7 days respectively

Translating the dose from a singular ingestion rate of 1 whole apple is captured in Eq. 5 Accounting for the aforementioned growth during storage before and after purchase yields a concentration of *L.*

monocytogenes within the stem cavity after processing, as shown in Eq. 6. Assuming the puncturing stage allowed for a perfect embedding of the surface area of the stem cavity into the stem cavity, Eq. 7 was used to calculate the concentration of *L. monocytogenes* on the surface of the apple pre-puncture, where *S* is the average surface are of an apple. It was assumed that the apple surface is homogenous, and that the pathogen was uniformly distributed on the apple surface pre-puncture.

$$C_{Exposed} = \frac{\text{Dose}}{\text{Ingestion Rate}} \quad (5)$$

$$C_{STEM} = C_{Exposed} - Growth \quad (6)$$

$$C_{APPLE} = \frac{C_{STEM}}{S_{STEM}} \, x \, S_{APPLE} \quad (7)$$

3.2.4.2 Chemical Interventions

There is minimal literature available characterizing common industry practices and the magnitude of shared practices when it comes to apple packinghouse facilities. Nevertheless, there are two common chemical interventions that are used across fresh produce packinghouses: Peracetic Acid and Chlorine. Both interventions have been investigated for their efficacy on apples through the treatment process at various concentrations, durations, and consideration of excess biological materials carried from the field within the apple's storage bins. A study by Su et al. (2022) quantified the log-reductions assumed to occur during processing within this rQMRA. The experiment was designed to evaluate the change in chemical concentration and treatment efficacy over time due to changes in initial concentration and the inclusion of other parameters such as superfluous biological products found within the dump tanks that are carried over from the field. The study evaluated a range of Critical Oxygen Demand (0-1000 ppm), concentration ranges of PAA (0-80 ppm), concentration ranges of Chlorine (0-100 ppm), and exposure times ranges (0-30 minutes), and simulated dump tank water (SDTW) to capture the efficacy of interventions on *Listeria monocytogenes* (Peneau, S., et al., 2006).

For this rQMRA, a uniform distribution of efficacy for the inventions was applied to account for all the variability within the environment during the simulations where the range of efficacy identified were 0.87 to 1.14 and 0.83 to 1.73 for Chlorine Reductions (R_{Cl}) and Peracetic Acid Reduction (R_{PAA}), respectively. A sensitivity analysis was conducted to evaluate the impact of this assumption. For comparison to international standards the concentration then needs to be considered per mass of an apple to produce the results in CFU per g (CFU/g) as seen in Eq. 8 and 9.

$$C_{APPLE-LOG} = ln(C_{APPLE}) + R_{Cl} + R_{PAA} \quad (8)$$

$$C_{PRE-TREATMENT} = \frac{e^{C_{APPLE-LOG}}}{m_{apple}} \quad (9)$$

Table 3: Exposure Assessment Parameter Table for Reverse Quantitative Microbial Risk Assessment

Variable	**Variable**	**Value**	**Distribution**
Surface Area, stem cavity (cm^2)	SA_stemcavity	[Mean = 125.13, SD = 1.8]	NORMAL
Surface Area, apple surface (cm^2)	SA_apple	[Mean=200.29, SD=35.42]	NORMAL
Mass of apple (g)	apple_mass	[Mean=177, SD=10.1]	NORMAL
Chlorine-Microbial Reduction (Log CFU)	R_Cl	[0.87,1.14]	UNIFORM
Peracetic-Acid Reduction (Log CFU)	$R\_(PAA)$	[0.83,1.73]	UNIFORM
Short-term Room Temp Growth (after 2 days)	GTr-S	[Mean = 2.5, SD = 2.1]	NORMAL
Extended-term Room Temperature Growth (after 9 days)	GTr-E	[Mean = 4.6, SD =2.62]	NORMAL
Extended-term Cold Storage Growth (after 2 days)	GTc-S	[Mean = 1.6, SD = 1.2]	NORMAL
Short-term Cold Storage Growth (after 9 days)	GTc-E	[Mean =3.0, SD = 1.87]	NORMAL

3.3 Results

3.3.1 Results of Dose-Response:

Point estimates of the risk of death and risk of stillbirth from the outbreak data were used to calculate exposure doses for the populations of interest using Equations 3-4. For the immunocompetent adult model, the median exposure dose was estimated to be approximately 20,000 CFU. For the stillbirth model, the median exposure dose was estimated to be 10 CFU. These exposure doses and the following exposure assessment models were used to calculate the concentration of *L. monocytogenes* on or in the apple within the apple packinghouses.

3.3.2 Results of Exposure Assessment

The concentration per gram of apple at the point of ingestion was calculated with the aforementioned exposure doses and an assumed average ingestion volume of 177 g of apple for a singular exposure event. Equations 5-9 and the parameter distributions described in Table 2 were used to calculate the concentration of L. monocytogenes on or in the apples prior to treatment. Concentration ranges dependent on consumer and retailer handling practices were produced and can be seen below in Table 4.

Table 4: Statistical summary of the Concentration of *L. monocytogenes* (CFU/g) in apple packinghouses pre-processing

	Temp, Time	2.50%	Median (50%)	97.50%
Healthy Adult	25°C, 2 days	0.5	9.8	357.0
	25°C, 9 days	< 0.01	2.3	138.0
	7°C, 2 days	2.6	19.8	221.0
	7°C, 9 days	0.5	7.0	166.0
Stillbirth	25°C, 2 days	0.07	1.41	39.60
	25°C, 9 days	<0.01	0.33	16.90
	7°C, 2 days	0.45	2.78	23.80
	7°C, 9 days	0.08	1.01	19.90

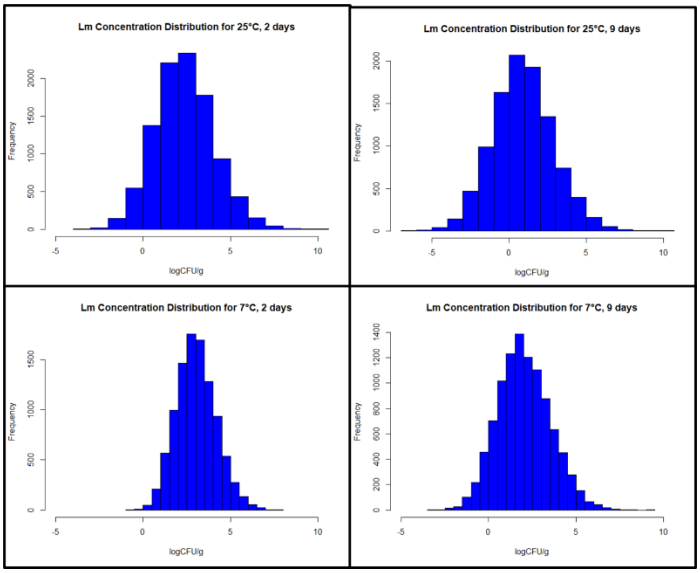

Figure 13**:** Immunocompetent model result distributions of concentrations of *L. monocytogenes* within apple packinghouses associated with the 2014 caramel apple outbreak based on consumer handling and retail storage practices. Each analysis of the consumer growth scenarios for short-term room temperature, extended room temperature, short-term cold storage, and extended cold storage

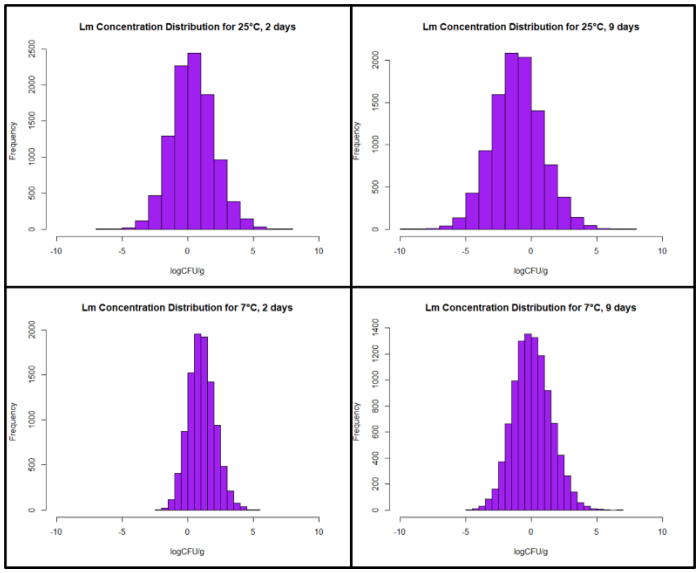

Figure 14**:** Pregnant-Stillbirth model result distributions of concentrations of *L. monocytogenes* within apple packinghouses associated with the 2014 caramel apple outbreak based on consumer handling and retail storage practices. Each analysis of the consumer growth scenarios for short-term room temperature, extended room temperature, short-term cold storage, and extended cold

As illustrated in the figures above, both models predict a low-level concentration after Monte Carlo simulations, of *L. monocytogenes* present in or on the apple prior to treatment. In Figure 13, the four growth scenarios resulted in similar ranges of concentrations with means of 58.1, 24.3, 42.0, and 27.2 CFU per gram of apple for the storage at 25°C (2d, 9d) and 7°C (2d,9d), respectively. The immunocompetent model for the average adult produced ranges of no more than 357 CFU/g at the 95% confidence upper bound and a minimum of all scenarios at 0.152 suggesting a low-level concentration of the pathogen associated with the documented outbreak. In Figure 14, the stillbirth model's room temperature extended storage (25°C, 9d) indicated a concentration level per gram of 0.008 CFU/g. With the stillbirth model, the four growth scenarios had medians of 1.41,0.33,2.78, and 1.01 CFU/g of apple for the storage at 25°C (2d, 9d) and 7°C (2d,9d), respectively.

3.3.3 Sensitivity Analysis

For this risk assessment, numerous assumptions were made to attempt to describe the concentrations of *L. monocytogenes* associated with the 2014 caramel apple outbreak. To investigate the impact of these assumptions, Spearman rho correlation coefficients were calculated. The highest correlation in all models is associated with the chemical reduction for Peracetic Acid. The findings of the risk characterization element can be seen below in Table 5.

Table 5: rQMRA sensitivity analysis with spearman rho correlation values (Concentration Output ~ Parameter Input)

Model	Temp, Time	Stem Surface Area	Whole, Apple Surface Area	Apple Mass	Chlorine Treatment	Peracetic Acid Treatment	Growth
Healthy Adult	25°C, 2 days	-0.03	0.38	-0.01	0.04	0.13	-0.90
	25°C, 9 days	-0.03	0.24	-0.02	0.04	0.13	-0.96
	7°C, 2 days	-0.06	0.60	-0.01	0.07	0.22	-0.72
	7°C, 9 days	-0.05	0.36	-0.01	0.07	0.17	-0.89
Stillbirth	25°C, 2 days	-0.01	0.25	-0.01	0.04	0.14	-0.95
	25°C, 9 days	-0.02	0.12	-0.02	0.04	0.13	-0.98
	7°C, 2 days	-0.05	0.44	-0.02	0.08	0.25	-0.82
	7°C, 9 days	-0.04	0.22	-0.01	0.07	0.18	-0.94

From the sensitivity analysis, most of the parameters show a limited relationship with the model output; however, the growth parameter for every model seems to demonstrate an elevated level of impact. For the Cold-short storage, the correlation seems to be significantly lower when compared to the correlation for other models which could align with findings in (Salazar, J.K., et al., 2016).

3.4 Conclusions & Implications

The findings in this study were able to showcase the potential for low-level concentrations in apple packinghouses. Through the construction of the mathematical model that was used limitations were identified and assumptions were utilized to support the development of the model. Once critical assumption in the models would be the interpretation of the epidemiological study. For the immunocompetent model, 35 people were assumed to be the population affected; however, this estimate is conservative as more individuals could have been impacted and gone unreported. Utilizing the endpoint of death does provide

more concrete values compared to considering the amount infected as the number infected shares similar problems in which capturing the true infected population probed questions of symptomatic or non-symptomatic, demographics, etc. Thus, death was the appropriate endpoint to base the model from as the binary nature of death statistics provided the most accurate adverse human health statistics. For the stillbirth model, the 11 pregnant women utilized is a more accurate number than the immunocompetent model also for the same reasons, the number of pregnant women exposed is more likely to contain nearly all the infected population that are pregnant. Comparing the two models, the stillbirth model provides a more accurate reflection of the adequacy of the current limit of detection than the immunocompetent results for this reason.

Generally, there is a lack of quantitative concentration data on the pathogen within apple packinghouses and food manufacturing facilities to support modeling such as growth of *Listeria monocytogenes* in food manufacturing environments, chemical intervention efficacy, etc. To account for this in the model, assumptions were made to construct the reverse risk assessment. In the apple exposure assessment, growth of *L. monocytogenes* was assumed negligible below the 4 ℃ in stages such as the Controlled Atmosphere room and during transportation. During processing, chemical interventions were applied a uniform distribution to account for the minimum and maximum within the Monte Carlo simulations. With knowledge from industry leaders, items such as industrial grade soap and the fan drying were able to be assumed negligible impacts as the efficacy and the temperature of the fan (65 °F - 85 °F) were identified to not have much impact on the reduction of *L. monocytogenes*. The sensitivity analysis identified the Peracetic Acid intervention to have a high correlation to the model's *L. monocytogenes* estimates suggesting that more refined modeling of the efficacy of this treatment may be needed This could include more in-depth exploration of numerous factors that impact the process such as concentration, Critical Oxygen Demand, extra biological debris, etc. The model did not consider recontamination of surfaces such as brushes and bristles within the process that could also be considered in future risk assessments. The work completed here can be utilized to investigate the behaviors of *L. monocytogenes* within these industries through comparative risk prioritization of storage practices, microbial persistence

modeling, and development of novel food safety interventions. The implications of the findings in respect to policy and practice can be seen in Chapter 4 (Future Work).

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Chapter 4: Connecting Research to Practice

4.1 Implications of Findings

This body of work looked to investigate the potential for low-level concentrations in apple packinghouses from the context of the 2014 Caramel Apple Listeriosis outbreak. The results of the reverse Quantitative Microbial Risk Assessment (Chapter Three) indicate the potential for gaps in current food safety technology and their limit of detections. Despite the United States preferring to sample the apple packinghouse environments as opposed to the RTE apples, the findings look at concentrations of *Listeria monocytogenes* on the apple itself. By looking towards international allies in Canada and Europe, the quantitative threshold in their food safety guidelines were used as these methods look towards directly sampling the apples. In Canada, the guidelines recommend <100 CFU/g for RTE foods, such as apples (Health Canada, 2023). Many European countries (France, Germany, etc.) have adopted a similar tolerance of 100 CFU/g, which is in alignment with the recommendations from The European Food Safety Authority (EFSA) (EFSA Panel on Biological Hazards [BIOHAZ], 2018). Another up-and-coming limit of detection to consider would be those of rapid biosensors specific for *Listeria monocytogenes* as these devices often are associated with low-costs, rapid results, and the ability to be integrated into Environmental Monitoring Programs (Justino, C.I., et al., 2017; Rodriguez-Mozaz, S., et al., 2005; Dennison, M.J., et al., 1995). Numerous varieties of biosensors exist for *Listeria monocytogenes* including optical biosensors (Geng, et al, 2004), Cell-based biosensors (Banerjee and Bhunia, 2010), and Amperometric biosensors (Davis et al, 2013). All of which provide sampling in CFU/g to allow for a comparison with the international policies as well. These all share a sampling detection of limit of 10^2 CFU/g, which is also shared in the policies in Canada and Europe (Soni, D.K., et al., 2018). With this understanding of the current limit of detection in technology and the threshold on RTE commodities from policy, the findings in the rQMRA were analyzed utilizing to observe how much of the low-level concentrations estimated would be below the limit.

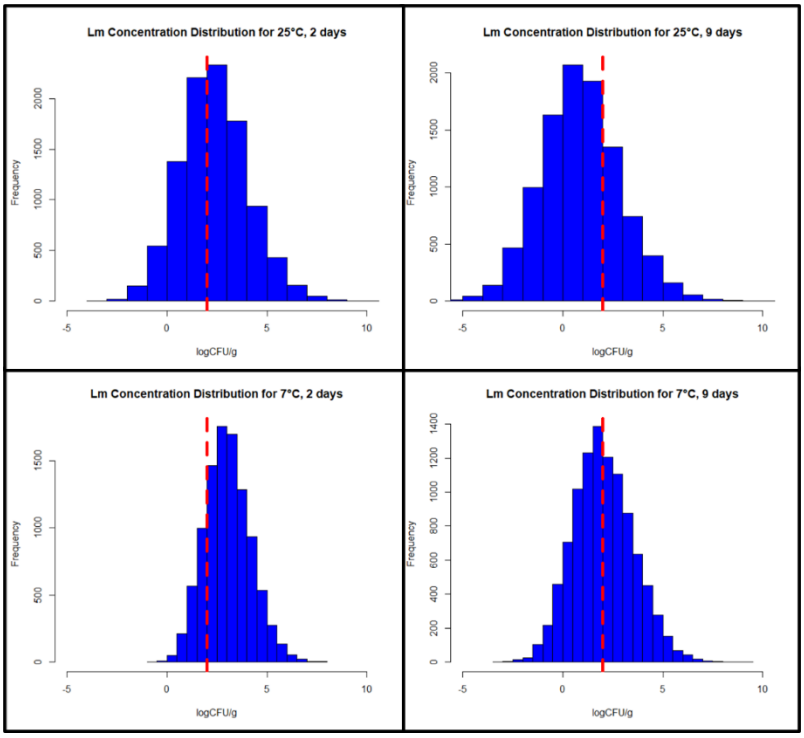

Figure 15: *Listeria monocytogenes* distributions from the rQMRA by each growth scenario with the applied limit of detection for modern policy and biosensor detection applied. The red dashed line indicates the current 2-log CFU/g limit for rapid biosensors and 100 CFU/g policy threshold for the immunocompetent model

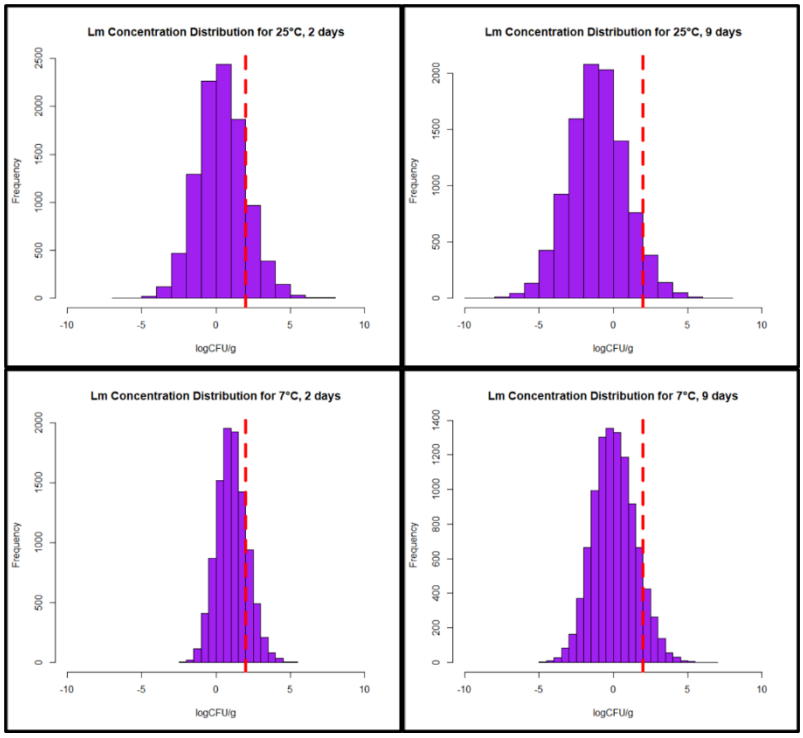

Figure 16: *Listeria monocytogenes* distributions from the rQMRA by each growth scenario with the applied limit of detection for modern policy and biosensor detection applied. The red dashed line indicates the current 2-log CFU/g limit for rapid biosensors and 100 CFU/g policy threshold for the stillbirth model

The 10,000 iterations of simulations allow for random selection within the distributions to provide 10,000 potential simulations for the potential low-level concentrations. From these 10,000 potential scenarios, Table 7 below highlights the percentage that would fall below this current quantitative threshold in policy and biosensors at 100CFU/g on the pretreated RTE foods.

Table 6: Results from the Immunocompetent model and Stillbirth model with applied 100 CFU/g limit of detection to identify the percentage of simulations underneath this limit

Model	Temp, Time	Percentage under Detection Limit (100 CFU/g)
Healthy Adult	25°C, 2 days	43.0%
	25°C, 9 days	72.8%
	7°C, 2 days	18.3%
	7°C, 9 days	51.6%
Stillbirth	25°C, 2 days	**84.6%**
	25°C, 9 days	**94.2%**
	7°C, 2 days	**82.3%**
	7°C, 9 days	**90.7%**

The immunocompetent model shows estimations at a higher concentration, which is supported by the lower number of simulations that are resulting in concentrations beneath the limit of detection; however, for the more sensitive populations nearly 88% of all four of the growth scenarios on average is falling beneath the limit of detection. The implications for this suggest that concentrations in apple packinghouses on RTE apples could be allowing for false negatives during sampling. When considering the sensitive populations, with up to 94.2% of sampling falling underneath the LOD that the current monitoring practice may not be as health protective and could be a potential mechanism for mitigating future Listeriosis outbreaks in RTE commodities, such as apples.

4.2 Conclusions

This thesis presents two primary components: (i) data synthesis and project scoping of information relating to *Listeria monocytogenes*, apple production, and packinghouse handling practices as well as (ii) a risk assessment leveraging reverse Quantitative Microbial Risk Assessment (rQMRA) to estimate low-level concentration of *L. monocytogenes* on apples. In combination, these components can be used to

support the understanding of pathogenic contamination events and to support risk-based management strategies that can be used to mitigate the potential for human health exposures to *L. monocytogenes*. The results of the rQMRA also contain significant findings that are critical takeaways from this work:

- *L. monocytogenes* biological properties support its ability to persist in food handling environments causing concerns for apple packinghouses.
- In the United States, the "zero tolerance" policy and current environmental monitoring practices could be providing inaccurate results during sampling allowing for *Listeria monocytogenes* exposure to apple consumers.
- The findings in the reverse risk assessment suggest low-level concentrations impacting sensitive populations may not be captured up to 94% of the time with current detection limits.
- Results of the risk assessment indicate that there are knowledge gaps associated with missed sampling events with current zone specification, prevalence distributions, and food safety detection limits with current methods.
- To support future risk assessments and understanding of pathogenic survival in RTE produce environments, quantitative concentration data should be investigated.

4.3 Future Work

This work established a profile of possible *L. monocytogenes* concentrations undetected in apple packinghouses. Future work leveraging this data should include the development of a comparative risk tool to support evidence-based economic decisions for all national apple growers, including those here in Michigan, to maximize profit and minimize the risk of contamination. Relative risk and logistical regression methods can be used to explore qualitative findings on common practices in apple packinghouses to explore how process changes or improvements may impact risk. This would need be done in conjunction with a survey-based study to explore the common practices of Michigan apple growers to fully understand the scope of the choices they face when operating these apple packinghouses.

Future assessments of the current model should explore more robust and nuanced modeling efforts that use the findings in this study to investigate pathogenic persistence. Within the treatment environment, understanding how the pathogen moves during transport of the process and the fate of the pathogen during interventions would be crucial in narrowing the uncertainty associated with how this pathogen persists in the environment, not just on commodities but within the broader context of the packinghouse facility including fomite surfaces of concern for exposure.

Outside of risk science approaches, modern biological sensors could be used in these complex environments that provide the nexus for transportation of *L. monocytogenes* within these critical points in the apple handling processes. Especially considering the potential limitations of current detection limits, leveraging rapid detection sensors for environmental monitoring could also be ideal for supporting the development of best monitoring practices. Studies comparing rapid environmental detection methods and clinical sampling could also continue to motivate the change in policy and support the mitigation of exposure to the public.

The findings in this study inform the level of concentration potentially on apples when introduced into processing, but the study did not identify the source of contamination of the outbreak. While the potential of low-level concentration is alarming, more work is needed to determine the sources of *L. monocytogenes* within packinghouses. Prevalence studies have been conducted that indicate that high areas of concerns within packinghouses include Controlled Atmosphere Rooms and High Traffic Areas (Ruiz-Llacahuanga, B., et al., 2021). The selection and prioritization of sampling zones represents another current research gap. The sampling zones for environmental monitoring of *L. monocytogenes* are numbered based on proximity to the product; Zone 1 indicates direct contact and Zone 4 indicates an area outside of the main room. Currently, only Zones 2 and 3 in apple packinghouses can be tested with current policy allowing only for testing in areas of indirect fomite transmission (Dunn, L. L., et al., 2023). In conjunction with the potential for mis-sampling generally, sampling in these indirect zones also offers the opportunity for low-level concentrations of the pathogen to pass through current mitigation systems. Finally, with the current "zero tolerance" policy in conjunction with indirect sampling for the pathogen, current gaps in food safety

technology could also be promoting the ongoing problem of these outbreaks (Nagata, J., 2015). Advancement in food safety technology to innovate more precise measuring methods and tools would improve detection with pathogenic concentrations, especially with recent breakthroughs in rapid biosensors for *Listeria monocytogenes* (Välimaa, A. L., Tilsala-Timisjärvi, A., & Virtanen, E., 2015). These sensors can obtain concentration data of the pathogen in the field within 15 minutes of sampling, though many have relatively high detection limits compared to the current sampling methods in place: One such device utilizes a colorimetric sensing platform to detect *L. monocytogenes* up by 2-log colony forming unit/ml (CFU/ml) (Alhogail, S., et al., 2016). Understanding every component of sampling efforts (where to sample, how to sample, what to sample) risk mitigation strategies can be implemented with concentrated but effective efforts to continuing the prevent more deadly outbreaks from *Listeria monocytogenes.* By integrating these concepts of Environmental Monitoring, *Listeria monocytogenes* policies, and utilization of rapid, biosensors the work to continue to improve strategies for mitigation of *L. monocytogenes* can continue to be improved and refined to support the public health protection.

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