DEVELOPMENT AND IMPLEMENTATION OF FOOD SAFETY AND QUALITY INDUSTRY INTERVENTIONS IN ACADEMIA: THE NEED FOR PRACTICALITY FROM A BUSINESS PERSPECTIVE

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ABSTRACT

Food safety and quality are integral departments of any successful food business, and while research is constantly identifying possible tools (e.g. artificial intelligence or novel microbiological testing methods) to further efforts in these departments, tools created by academia need to be practical from a business perspective in order to be most effective and adopted into industry. To address this, three investigations were conducted. The first investigation explored whether novel AI and digital technologies are suitable for human pathogen control in Controlled Environment Agriculture (CEA) and the associated challenges and opportunities that could come with implementation in the industry. To achieve this, AI, food safety, and CEA experts gathered to conduct a gap and opportunities workshop for AI implementation for human pathogen control in CEA. Additionally, a site visit to a CEA business was conducted to view operations. Outcomes from this investigation highlight how tools created by academia for food industry, in order to be effective, need to take into account i) the industry's current level of food safety maturity, ii) current industry practices and norms and, iii) how to effectively communicate the quantitative financial benefit (i.e., in this case, reduction in recall risk) to business leaders. The second study involved the development of a food quality related tool specifically for the dairy industry: a microbiological test to enumerate quality defect causing thermoduric bacteria in dairy with a special focus on non-sporeforming bacteria. To address this, 38 isolates from non-sporeforming genera were inoculated into skim milk broth and independently subjected to four different heat treatments (A: 63°C for 30 minutes, B: 65°C for 15 minutes, C: 68°C for 7 minutes and D: 70°C for 5 minutes), followed by plating using two different media types (Standard Methods Agar and Aerobic Count Petrifilms), each of which were incubated and enumerated after three different incubation periods (24h, 48h, and 72h) at

32°C. All combinations were compared to our gold standard test, the Laboratory Pasteurization Count (i.e., heat treatment of 63°C for 30 minutes followed by plating with Standard Methods Agar and incubation of plates for 48h at 32°C). This study attempted to utilize knowledge gained from the first investigation such as use of current industry standard practices (i.e., we made sure to use standard dairy industry media [Standard Methods Agar] and an incubation temperature [32°C]). Interestingly, our results suggest that i) Aerobic Count Petrifilms are not a suitable media to enumerate some thermoduric bacteria and, ii) 72h incubation time presented a significantly smaller log reduction (i.e., there was more bacterial growth at 72h) compared to the gold standard incubation time of 48h. However, due to the small effect size in cell count reduction between the 48h incubation and 72h incubation (i.e., estimated mean pairwise difference in cell count reductions between 48h and 72h of only 0.28 log CFU/mL), the final method suggested for further exploration in this study utilized an incubation time of 48h. The researchers selected the 48h incubation time because, from a business perspective, waiting the additional 24h for marginally more exact results did not seem valuable for industry. Finally, the third study worked to address a gap highlighted in the first investigation; the need for identification of costs associated with implementing food safety tools for industry. Furthermore, it aims to understand what drives small and medium-sized businesses to invest into food safety programs as they may face unique challenges. This study involved using 9 small and medium-size dairy processing plants (SMDPPs), which all had been part of a prior ~1 year-long *Listeria* Environmental Monitoring Program (EMP) implementation study. Each plant was emailed an EMP cost questionnaire which was used to obtain each plant's (i) self-reported EMP associated costs (e.g., estimated total value of product in a plant at any given time [EVTFP], corrective actions costs) and, (ii) perceived ability to control pathogens in their processing environment.

Finally, plants were evaluated by the research team on their food safety culture resulting in a food safety culture score. Results showed that variables including plant size, EVTFP, overall *Listeria* prevalence in a plant, and food safety culture scores are not reliable predictors for how much a plant invested into its *Listeria* EMP indicating that investment into EMP for SMDPPs may be influenced by other, outside or non-food safety related factors. Overall, the outcomes from these three investigations illustrate that food safety and quality tools, in order to be most effective, need to take into account the industry's current food safety and quality maturity, work to utilize pre-existing foundations/standard practices in the industry, and communicate costs and financial returns to business leaders.

Biographical Sketch

Caroline Motzer was born in Seattle, Washington, on March 20th, 1997, to Bill Motzer and Lederle Tenney. Caroline lived in Seattle throughout her childhood with her two older brothers Charlie and Tom Motzer. She attended Seattle Country Day School (Elementary and Middle School), and Seattle Preparatory High School. During high school, she was a member of the Pocock Rowing Center for all four years, participating in boats that placed in the top three in the region each year as well as participating in Varsity Swim team and Cross Country.

Following her high school graduation in 2015, Caroline attended Cornell University, where she was a varsity rower for three years and joined the cheerleading squad in her senior year. She earned two B.S. degrees, one in International Agriculture and Rural Development and another in Agriculture Sciences, along with minors in Global Health and Soil Sciences.

After graduating from Cornell in 2019, Caroline worked as a hydroponic grower at a vertical hydroponic microgreen farm in Seattle. She then became a Food Safety Scientist and SQF Practitioner at a plant-based meat startup in Seattle. In 2022, she was accepted into the Master of Science program in Food Science and Technology, working in the Food Safety Laboratory and Milk Quality Improvement Program. Caroline hopes to take her skills to industry. Outside of academia, Caroline enjoys running, playing soccer, travelling, hiking, hanging with her cat Pip, and giggling with her friends.

I dedicate this thesis to my parents, Lederle Tenney and Bill Motzer. Dad, thank you for instilling in me a love of science and a sense of humor. Mom, thank you for teaching me empathy and a deep love for my friends and family.

I could not have done this without you both. I love you.

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CHAPTER I

INTRODUCTION

Food safety and quality are important goals in all food businesses, although they can be overshadowed in priority for more seemingly profitable departments such as sales or product development. Examples such as the 2008 Peanut Corporation of America *Salmonella* scandal in which company officials released product despite it testing positive for *Salmonella Typhimurium* in order to make sales can point to this. In response to the positive results, the company fabricated negative Certificates of Analyses (COAs) in order to continue shipments to customers (Goetz, 2013; United States Department of Justice, 2015) thus pointing to this problem of under-prioritization of food safety based on profits. In order to incentivize food businesses to implement new food safety and quality tools (e.g., Artificial Intelligence [AI], novel microbiological testing methods), it is imperative to understand how best to implement them into food businesses, where profits remain a top priority, so that interventions can be most effective.

One considerable emerging technology that could be applied to food safety in industry is that of Artificial Intelligence (AI) and emerging digital tools such as agent-based models and simulation models (Qian et al., 2023), however certain food industries (e.g., Controlled Environment Agriculture [CEA]) may be better suited than others to implement such technologies due to their reputation as being data intense. CEA is a rapidly growing sector (Grand View Research, 2023) and covers a wide span of production systems that vary in technology level such as “low tech” traditional greenhouse systems to “high tech” fully automated closed loop vertical farms (Benke & Tomkins, 2017; Engler & Krarti, 2021). CEA involves the use of specific agricultural practices and precisely controlled environments directed

towards the efficient growth of fruits and vegetables for consumption (e.g., leafy greens, tomatoes, strawberries) using limited resources (Benke & Tomkins, 2017; Engler & Krarti, 2021). The first chapter of my thesis addresses whether AI and digital technologies are an appropriate solution for microbiological food safety hazards in CEA. Additionally, this chapter identifies the challenges and opportunities associated with implementation of said technologies within the CEA industry. Essentially, it can be viewed as a case study on how to make a novel, effective food safety tool for industry. To do this, I met with CEA and AI subject matter experts from Wageningen University in the Netherlands and Centro de Edafologia y Biologia Aplicada del Segura (CEBAS) in Spain to host a workshop to discuss challenges and opportunities of utilizing AI to control microbiological food safety hazards within CEA operations as well as visit CEA operations in Spain to learn more about CEA-specific microbiological food safety hazards.

While CEA is a newer industry, there are still improvements of quality and food safety to be made in more established industries such as the dairy industry. For example, one such quality-related problem within the dairy industry is that of thermoduric bacteria, or bacteria that can survive temperatures considerably above their typical growth temperatures (Boor & Martin, 2024). Many of these bacteria can show limited reductions at typical pasteurization temperatures (R. T. Lee et al., 2024; Ranieri & Boor, 2009; Ribeiro Júnior et al., 2018) allowing them to bypass processing hurdles and implicate finished product. There are two main groups of thermoduric bacteria; endo-sporeforming bacteria (i.e., sporeformers) and non endo-sporeforming bacteria (i.e., non-sporeforming thermoduric bacteria [NSTB]) and both can cause quality related defects and decrease shelf life (Ribeiro Júnior et al., 2018; Washam et al., 1977). However, both groups differ in their thermal resistance, with sporeformers being able to withstand temperatures considerably higher than NSTB (Besten et al., 2018; Lindsay et al.,

2021). Sporeformers in their spore state are the primary group that survive HTST pasteurization (Ranieri and Boor, 2009), which involves heat treatment at a minimum of 72°C for 15 seconds. Conversely, only a small subgroup of NSTB can survive this process. Interestingly, the standard method used for enumerating total thermoduric bacteria, NSTB and sporeformers alike, in raw milk is the Laboratory Pasteurization Count (LPC), which involves only heat treating raw milk to 63°C for 30 minutes (a considerably lower temperature than HTST) followed by pour plating on Standard Methods Agar (SMA), and incubation for 48h at 32°C (Boor and Martin, 2024). A limitation of the LPC method is that it selects for a broad group of thermoduric bacteria (Lee et al., 2024) that do not align well with the populations found after the most commonly utilized heat treatments in the dairy industry (i.e., High Temperature Short Time [HTST] pasteurization). In other words, the LPC test captures bacteria that would not be able to withstand HTST pasteurization leaving a gap for improved thermoduric testing optimization with the goal of aligning the resulting microbial population to that which would more likely withstand HTST pasteurization. Therefore, the second chapter of my thesis highlights laboratory method development in comparison to the gold standard, the LPC test with a special focus on NSTB. An aspect of the desired method is that it can be very easily applied into the dairy industry as it stands currently, therefore, in addition to providing valuable results to the industry, the test needs to utilize equipment and media that dairy industry players are already familiar with. This portion of my thesis can be viewed as a case study of developing a tool with the goal that it will be used in a well-developed, pre-existing industry.

Finally, in order for adoption of food safety and quality tools to occur, interventions need to be financially practical as well. However, there is limited research regarding expected costs of food safety and quality interventions to the food industry, presenting a large gap in knowledge.

Challenges to this type of research revolve around the sensitivity of financial related data for businesses (Gomez & Marks, 2020) and, in terms of food safety, difficulty in quantifying financial returns that stem directly form a reduction in recall risk. Additionally, when characterizing costs of food safety interventions, size of businesses needs to be taken into account. For instance, small and medium businesses may face unique challenges when it comes to implementing food safety and quality interventions such as i) limited financial resources (Bhaskaran, 2006; J. C. Lee et al., 2023), and ii) possible underappreciation of food safety programs by small and medium businesses driven by poor food safety culture (Magiya, 2023; Nayak & Waterson, 2017). Furthermore, small and medium businesses, specifically dairy plants, have been implicated in recent listeriosis outbreaks and *Listeria monocytogenes* recalls highlighting the need to improve *Listeria* control programs such as *Listeria* environmental monitoring programs in small and medium dairy plants (SMDPPs) (Centers for Disease Control, 2007, 2017, 2022; Food and Drug Administration, 2023). To this point, the third chapter of my thesis is aimed towards i) understanding what drives SMDPPs to invest into *Listeria* EMPs, and ii) quantifying how much they are investing. This portion can be viewed as a proof of concept for research attempting to gather sensitive financial data from industry in order to evaluate costs of food safety and quality tools.

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CHAPTER II

FOOD SAFETY RELATED DATA ANALYTICS, DIGITAL, AND ARTIFICIAL INTELLIGENCE NEEDS AND OPPORTUNITIES IN CONTROLLED ENVIRONMENT AGRICULTURE*

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SUMMARY

Controlled Environment Agriculture (CEA) is increasingly used to grow food (namely fruits and vegetables) in controlled indoor conditions. While often billed as "eliminating" the classical food safety concerns associated with open field cultivation of produce, traditional as well as potentially novel microbial food safety risks are a concern for CEA, as supported by a recent salmonellosis outbreak in the US linked to CEA grown produce. In addition, the use of diverse technologies and practices in CEA represents a challenge in efforts to develop food safety guidance. CEA, particularly precision vertical farms, however, have the distinct advantage of being "data intense" and typically have a better data collection and management structure than is found in traditional agriculture. This may position at least part of the industry to use digital tools and AI to manage food safety. Possible AI approaches may include adaptive sampling and interventions depending on the presence of risk factors that could be predicted with the routine data generated during CEA operations. This article summarizes challenges and opportunities for using AI and digital approaches to assure microbial food safety and manage food safety related business risks in CEA.

OVERVIEW OF CEA

Controlled Environment Agriculture (CEA) is a rapidly growing sector (Indoor Farming Market Size, Share, Growth Report, 2030, 2023) due to its use of agricultural techniques to create specific and precise controlled environments directed towards efficient plant production using limited inputs (Benke & Tomkins, 2017; Engler & Krarti, 2021). CEA encompasses a wide array of technologies ranging from "low tech" traditional greenhouses to advanced and more automated soilless "high tech" and closed loop vertical farms. Currently, CEA production largely focuses on specialty crops (e.g., leafy greens, herbs, microgreens, tomatoes), but the term CEA

can also include indoor production of mushrooms, fish, and even insects (Cowan et al., 2022; Engler & Krarti, 2021; Govorushko, 2019). CEA is most commonly divided into soilless growing methods (such as hydroponics) and traditional growing methods (utilizing pots with soil) (Graamans et al., 2018). Both formats use various inputs, including growing media, lighting systems, and structural systems (Sardare & Admane, 2019). Most CEA systems are characterized by a wide array of high-tech operations which include common activities linked to the production of fruit, vegetable and herbs (FVHs) (e.g. seeding, irrigation, harvesting). An increasing number of CEA facilities also include processing and packaging operations. Operations such as cutting, washing and packing, which were usually performed in fresh-cut processing plants, thus are now performed within the CEA facilities, in the same room or adjoining rooms where the crops are grown (Hamilton et al., 2023).

Hydroponics is the technique of growing plants using a water-based nutrient solution and can include an aggregate substrate or growing media such as vermiculite, coconut coir, perlite or peat moss (Misra & Gibson, 2021; Sanjuan-Delmás et al., 2020; Savvas & Gruda, 2018). Hydroponics differ from traditional soil cultivation methods in that the water is the primary nutrient carrier as opposed to soil; the structural support is offered through the aforementioned substrate or growing media instead of soil. The most common types of FVH commodities cultivated under soilless practices are tomatoes, peppers, lettuce, and other leafy greens, including microgreens. Hydroponics include many different types of cultivation systems such as deep-water culture hydroponics, aeroponics, and aquaponics (Velazquez-Gonzalez et al., 2022). Deep-water culture is where seedlings are planted into floating rafts so that the roots are immersed in deep, recirculating nutrient rich “ponds.” In aeroponics, the roots of the plants are suspended in the air, and water and nutrients are supplied to the plant through a fine mist

activated by a timer. Indoor aquaponic systems combine plant production with fish cultivation, using treated water from the fish tanks as a source of irrigation water to grow plants (Palm et al., 2018). While hydroponics is inherently different from soil-based methods, the overall primary inputs for both methods, which are all relevant to food safety, remain generally similar. These primary inputs include plant seeds, water, nutrients/fertilizers, soil (for traditional methods) and substrates (for soilless methods) (Dankwa et al., 2020). Other food safety relevant factors in CEA production include lighting, structural systems and climatic factors (e.g. oxygen, humidity, and carbon dioxide levels) (Dankwa et al., 2020; Ilic et al., 2017).

Lighting systems involved in CEA cover a wide range. Traditional methods utilize sunlight, as commonly seen in greenhouses (Bo et al., 2023). On the other end of the spectrum are vertical farms where layers of crops are stacked on top of one another, each layer with its own set of lights (typically light emitting diode [LED]) on the bottom of the above structure (Engler & Krarti, 2021). This allows for precise dosing of lights, and often very defined amounts of red, blue and white light are utilized to increase plant production (Lin et al., 2013).

Finally, there can be numerous combinations between light and cultivation systems, each combination affecting the structure within the greenhouse/farm. Both hydroponics and traditional soil-based cultivation can be applied into a flat, one tier system using sunlight (Grewal et al., 2011). Hydroponics can also be applied into a multi-level vertical farm using synthetic lights (Engler & Krarti, 2021). It is unlikely that traditional soil methods will be combined with vertical farming as the weight of the soil poses a challenge for the structure to support. Two commonly seen types of CEA facilities are high tech vertical hydroponic farms utilizing LED lights and middle to low tech hydroponic horizontal farms utilizing sunlight. The latter system, which includes a hydroponic system, with several automated stages of the whole process (e.g. transport

of trays/beds from the seeding room to the greenhouse and from the greenhouse to the harvest area), is becoming increasingly popular (Hamilton et al., 2023). While this lower tech version may not have full climatic control, it might reduce the initial investment, making it attractive for firms (Grewal et al., 2011).

The range of practices in which environmental factors such as temperature, light, humidity, oxygen, and carbon dioxide are controlled can often be tied with the maturity of the data collection infrastructure. On one end of the spectrum, some facilities have tight control with constant measurements, allowing for precise changes to be made. On the other end are greenhouses with no formal data collection. In this instance, changes to the environment are based on knowledge from a grower. This poises some facilities to be more easily adapted to the use of AI technology than others, based on their data collection infrastructure.

OVERVIEW OF CEA FOOD SAFETY CHALLENGES

CEA is often framed as reducing or even eliminating traditional microbial food safety hazards due to the physical protection of the crop from the environment (Sela Saldinger et al., 2023), which is expected to lead to a lower likelihood of the edible part of the produce being in contact with wild animals and animal feces (Koutsoumanis et al., 2023). Supporting this, several authors have found lower total bacterial counts and lower microbiota diversity in crops grown in CEA systems compared to those grown in open fields (Gomes Neto et al., 2012; Williams & Marco, 2014). However, CEA clearly cannot produce products with "zero risk" of causing microbial or other food safety issues (Zwietering et al., 2021) and typical foodborne pathogens and perhaps unknown or waterborne pathogens are still a possible issue. Likely microbial food safety challenges in CEA include (i) pathogen contamination of inputs (e.g., seeds, substrates), (ii) pathogen contamination and persistence in water and water associated infrastructure, (iii)

pathogen contamination and persistence in the overall facility infrastructure (e.g. conveyor belts, harvest equipment, trays/beds, floors), and (iv) risk of pathogen transfer throughout the system; due to the interconnectivity of hydroponics via water and general lack of full system sanitation, there is the regulatory issue of lot separation. Food safety risks in CEA are supported by a salmonellosis outbreak in the US in 2021, which was linked to CEA (specifically hydroponically) grown prepackaged salads (Center for Disease Control, 2021). While the outbreak strain was not recovered from inside the facility (it was found in an "outdoor storm water drainage pond beside the farm" (Center for Disease Control, 2021), the investigation of this outbreak did detect another *Salmonella* strain (serovar Liverpool) in an indoor pond that was used to grow lettuce. This illustrates that *Salmonella* represents a hazard that needs to be controlled and addressed in CEA agriculture. Additionally, in January of 2024, a recall of CEA (specifically greenhouse) grown prepackaged salads was reported due to possible contamination with *Listeria monocytogenes* (Food and Drug Administration, 2024). A positive result for *L. monocytogenes* was identified during routine product testing. At the time in which this paper was written, no illnesses have been reported. Another microbial hazard that would need to be addressed (due to its presence in a wide range of sources, including natural environments), at a minimum, is enterohemorrhagic *Escherichia coli* (EHEC). Supporting the range of possible food safety hazards in produce, *Salmonella*, *E. coli* O157:H7, human noroviruses and *L. monocytogenes* have all been identified on hydroponic produce (Lopez-Galvez et al., 2014; Orozco et al., 2008; Shaw et al., 2016). While prevalence studies of foodborne pathogens have been performed in the production environment of open fields, as well as packinghouses and the processing environment of fresh-cut facilities (Belias et al., 2021; Castro-Ibáñez et al., 2016;

Sullivan & Wiedmann, 2020), more research to systematically assess food safety risks in the growing and harvesting of leafy greens under CEA systems is needed.

In addition to known foodborne pathogens, it also needs to be considered that pathogens that are not typically considered foodborne, as well as unknown pathogens, could be transmitted through CEA grown crops. Of special concern are emerging waterborne hazards (e.g. *Legionella*) that may be present in various water sources, which might be applied in CEA systems (Koutsoumanis et al., 2023). Particularly relevant is the recirculation of the nutrient solution in the hydroponic system. Water sources, such as municipal water, reclaimed water, and surface water, present differing levels of associated microbial risks, as each source varies in treatments and testing requirements, with municipal water typically considered the lowest risk (Gómez et al., 2019). Additionally, irrigation distribution networks can affect the water's microbial quality. For example, intermittent water supply (akin to the ebb and flow hydroponic technique) in India was found to have higher total coliform and *E. coli* counts at taps compared to levels taken from taps supplied with constant water (Kumpel & Nelson, 2013). Whether the system or equipment is temporarily or permanently assembled, the methods by which water storage, delivery, and distribution systems are cleaned, maintained, and stored are crucial. Given the range of possible microbial contaminants possible in CEA, more specific research clearly identifying microbial hazards and their frequency is needed for the CEA sector.

While growing media for hydroponics, aeroponics, aquaponics or soil-based growing systems vary widely and can provide avenues for contamination of final product, there is still limited research that would help with an assessment of risks associated with different inputs. Nutrient solutions, non-synthetic fertilizers (fish emulsion, algal extracts, liquid green waste extract), and non-soil growth media such as coconut coir or perlite are all examples of inputs

used in CEA. Different CEA systems typically have specific requirements for handling of inputs to avoid cross-contamination. Notably, CEA that integrates fish (aquaponics) has a potential source of fecal contamination built into the production system. While there is some research regarding plant pathogens in soilless inputs (Calvo-Bado et al., 2006; Clematis et al., 2009; Lopez-Galvez et al., 2016; Stanghellini & Rasmussen, 1994), there is little research regarding the human pathogenic prevalence of CEA inputs. One study (Dankwa et al., 2020), illustrated the role of substrates as a potential source of contamination in hydroponic systems, which can facilitate microbial transfer to harvested leaves. Similarly, Işık et al. (Işık et al., 2020) found that growth media used in soilless microgreen production can affect the transfer of pathogens to edible and inedible portions of microgreens. Due to the lack of knowledge surrounding the microbial environment of these inputs, combined with the wide range of inputs used and the different systems they are used in, there is a large knowledge gap regarding human pathogen microbial risks from inputs how inputs facilitate the spread to edible or inedible portions of products (Hamilton et al., 2023).

While automated systems can control seeding, planting, moving plants, irrigation (e.g. timed release and dosing of water and nutrients), harvesting, and post-harvest processing (e.g. moving growing trays pulled along a series of chains and pulleys, packing), these automated systems are often not hygienically designed or created with sanitation/disassembly in mind. This, combined with year-round production and limited sanitation (e.g. once a year), increases the potential risk of persistence and spread of pathogens. In addition, complex equipment and infrastructure in CEA may require extensive and well-managed maintenance to minimize food safety risks. In vertical farms, there are permanent structures with multiple growing levels. Equipment associated with growing includes multiple levels using shelving systems or vertically

mounted growing systems (e.g., growing troughs suspended from the ceiling) and vertical conveyor systems (Engler & Krarti, 2021). Relevant to vertical farms, if any of the top layers become contaminated, there is a high likelihood that it could disperse via water droplets onto lower levels and onto edible portions of plants. Future research and development on hygienic design and sanitation in CEA facilities (including validation and verification) thus would be valuable (Hamilton et al., 2023).

An additional challenge associated with microbial food safety in CEA is that there is limited knowledge of the microbiome within a CEA facility compared to a traditional agriculture microbiome. Plant microbiomes have been reported as playing important roles in securing food production and reducing microbial food safety risks (Callens et al., 2022). For example, sensitivity to invasion by pathogens can be characterized by different states of plant microbiomes, i.e., the ones that are in dysbiosis and therefore sensitive to pathogen invasion vs. the ones that are in eubiosis and more resilient to microbial perturbations (Berg & Cernava, 2022). Microbial interconnectivity between ecosystems plays an important role in the development of plant microbiomes and microorganisms can be transmitted via the internal compartments of seeds to mature plants (Sessitsch et al., 2023). Also, human pathogens can be transmitted via seeds to growing plants, although internal contamination with pathogens appears to be unlikely to occur (van Overbeek et al., 2020). A scientific opinion published in 2011 (2011) hypothesized that the increased proliferation of inoculated Shiga-toxin producing *E. coli* (STEC) on hydroponically-grown microgreens could be potentially due to a less competitive microbiota. Hence, further research of the plant and overall environmental microbiome in CEA facilities, and the impact of microbiomes on food safety risks in CEA systems, may be valuable.

A specific operational challenge is that production in CEA facilities is essentially continuous, which means that it is typically difficult for facilities to define and validate a "clean break" between production lots. Supporting this, researchers hosting a two-day conference regarding food safety in CEA titled Strategizing to Advance Future Extension and Research (S.A.F.E.R.) in April 2023 identified a lack of clean breaks as a challenge that could be potentially mitigated with the use of artificial intelligence (AI) (Hamilton et al., 2023). While there is no clear path to defining a clean break without full sanitation, AI-facilitated data analytics may be able to use regular (e.g., daily) testing data, as well as other data (e.g. sampling effort), to characterize the likely length of a contamination event although it is possible this could be underestimated due to the inherent rare nature of contamination events. This would be valuable as a lack of a break between lots can represent a substantial business risk as regulatory agencies (as well as customers) may, in case of a single contamination event (e.g., a product sample collected on a given day that tested positive for a pathogen), request and/or require clean breaks to agree on a recall that is limited to one day's production. Without a sanitation break to support that contamination would not have been carried over to subsequent days and lots (e.g., through water), it may be necessary to issue a recall that covers all products in the marketplace (Chapman & Danyluk, 2013). In some CEA facilities, production is only stopped once a year (which is when repairs and equipment cleaning occur); even then, full clean breaks may not occur, e.g., if water is not completely removed in deep water hydroponic systems.

Because CEA is a unique position between both primary producer and processer, the regulatory framework applicable to CEA is often unclear, leading to gaps in regulation and confusion for both regulators and industry. There however are already specific guidelines describing Good Agricultural Practices (GAPs) and Good Hygiene Practices (GHPs) for CEA

facilities (Food and Agriculture Organization of the United Nations, 2017), which include general recommendations such as: (i) protected facility structures should be located, designed and constructed to avoid contamination and harborage of pests; (ii) worker training and sanitation practices are necessary in all facilities; (iii) proper water management and soil amendment use are critical to controlling and reducing risks. Additionally, in the US, CEA firms are expected to adhere to the Food Safety Modernization Act (FSMA) and the Produce Safety Rule (Food and Drug Administration, 2023a). That being said, CEA is unique compared to the traditional produce supply chain because growing, harvesting, processing and packaging often occur in the same room or adjoining rooms, leading some firms to additionally be governed by the Preventive Controls for Human Food Rule (Food and Drug Administration, 2023b) depending on their system. This can cause confusion from regulatory bodies and industry about what recommendations and frameworks apply, potentially leading to gaps in food safety and sanitation systems (Hamilton et al., 2023). A related challenge is that CEA needs more guidance, as well as field-based assessments and data-based development of corrective actions, mitigations, responses to positive findings, including standardization of environmental monitoring programs (EMPs) for CEA (Allende et al., 2023; Suslow et al., 2019). A specific challenge for EMPs in CEA is that it may be hard to distinguish between the different Zones, which define proximity to food (i.e., Zones 1, 2, 3, 4) (Spanu & Jordan, 2020), as essentially all surfaces are connected via water recirculation. Formal risk assessments or risk ranking may thus be needed to rank food safety risks associated with CEA of specialty crops and to inform food safety regulations for CEA (Hamilton et al., 2023).

Finally, in all food processing environments using human intervention, microbial and viral contamination via employees is a possible source. Personal hygiene and health

requirements are critical as CEA personnel come directly in contact with edible and inedible portions of FVHs during seeding, and harvesting/packaging (Bihn & Reiners, 2018). In many CEA facilities, Good Manufacturing Practices (GMPs) or GAPs are enforced, such as personal protective equipment (PPE) wearing and hand washing (Misra & Gibson, 2021); however, in some CEA facilities there may be a need to strengthen these practices.

OPPORTUNITIES FOR DIGITAL AND AI TOOLS IN CEA FOOD SAFETY

There have been a number of exciting advances in the food safety applications of AI and digital tools, spanning both mechanistic (e.g., simulation and agent-based models) and data-driven approaches. Some of these tools have taken advantage of new real-time data streams, such as social media and mobile apps, or have benefited from other large scale data streams and technologies, such as next-generation sequencing (NGS), smart labels, and blockchain. The development of sensors and their integration with Internet of Things (IoT) technologies also provides an avenue for data streams that could be utilized by AI to manage food safety risks (Miranda et al., 2019). The application of these AI and AI-enabling technologies has shown promise in different areas, such as better pathogen detection and disease control. For example, BERTweet extracts foodborne illness-related entities from Twitter/X and was shown to identify unreported foodborne illness outbreaks (Tao et al., 2021). A Bayesian hierarchical model was developed for real-time monitoring and nowcasting of foodborne disease cases from public health surveillance data (Wang et al., 2018). Using *L. monocytogenes* in milk as an example, Njage at al. (Njage et al., 2020) demonstrated how machine learning (ML) can be used to predict stress phenotype components for new unknown pathogen strains given their whole genome sequencing (WGS) data, which could improve risk assessments for foodborne pathogens. Agent-based models (ABMs) developed to recreate a specific food facility, have provided facility-

specific “personalized” food safety decision support for the food production environments (Zoellner, Jennings, et al., 2019). A study by Nogales at el. (Nogales et al., 2022) demonstrated the utility of neural models in optimizing the number of food safety inspections using data from the Rapid Alert System for Food and Feed (RASFF), which facilitates the exchange of information about health threats in European countries. Importantly, a number of peer-reviewed studies have developed and detailed specific tools that can be applied to CEA, including simulation-based and ML tools that can be used to predict food safety risks and facilitate improved control. For example, a series of papers (Barnett-Neefs, Sullivan, et al., 2022; Barnett-Neefs, Wiedmann, et al., 2022; Sullivan et al., 2021; Zoellner, Jennings, et al., 2019) has detailed how ABMs can be developed and used to facilitate improved control of environmentally transmitted foodborne pathogens. Also, a simulation model was developed to assess the listeriosis risk associated with a contaminated production lot of frozen vegetables (Zoellner, Wiedmann, et al., 2019) as an example of a decision-support tool for food safety and business management that can also be applied to CEA. Similarly, a number of different decision tree and ML-based methods have been applied to predict times and locations with an increased risk of pathogen contamination in fields and water sources (Strawn, Fortes, et al., 2013; Strawn, Gröhn, et al., 2013; Weller et al., 2015, 2016, 2016); these approaches could also be adapted to CEA, even though input data would obviously be very different.

One key opportunity for digital and AI tools in CEA will be to integrate AI-based and digital food safety prediction and management tools into the overall digital infrastructure for CEA (i.e., systems that manage temperature, lighting, etc.). The ultimate goals of these efforts would be to (i) reduce food safety risks and manage them more effectively, (ii) minimize food safety-associated enterprise risks (e.g., by developing and implementing systems that can provide

for validated “clean breaks” between lots), and possibly to (iii) provide enhanced transparency of food safety efforts to customers and possibly regulatory agencies. While these efforts will require (i) robust data acquisition systems and (ii) custom-tailored AI and digital tools, as detailed in the section above, existing tools that represent a starting point for these efforts already exist. Ultimately, proactive food safety systems for CEA could benefit from the development of comprehensive “digital twins” (Defraeye et al., 2021), which would be individualized to each facility and be driven by real-time data to predict food safety risks. These digital twins could then be used to adjust food safety measures (e.g., testing frequencies, sanitation procedures, water treatment) to better control food safety risks and better manage situations with increased risk of pathogen contamination. Additionally, these models can also be complemented by various imaging or spectral AI models to identify hot spots for organic build-up on diverse surfaces and Zones in CEA facilities (Feng & Sun, 2012). A connection between AI models and microbial testing results can further aid in enhancing the relevance of these models for the assessment of food safety risks. This would allow CEA facilities to understand how different environmental and structural factors interact and affect the probability of foodborne pathogen (e.g. *Salmonella* and *L. monocytogenes*) contamination in different CEA facilities, and potentially reduce the cost of microbial sampling. Finally, these digital twins could be used to concurrently manage food safety as well as other outcomes such as quality, productivity etc., which could be used to optimize trade-offs between food safety, productivity, and quality. An example of such a trade-off is related to the negative impacts of high levels of chlorine that may be used, at the cost of quality, for water treatment and management of food safety risks. Additionally, combining models to manage food safety as well as quality and productivity will make it a more attractive product for the industry to implement.

More targeted food safety-related applications of AI in CEA could address the unique food safety challenges posed. Primary inputs (e.g., substrate, water, seeds) present a pathway of contamination in CEA, so the development of AI approaches based on imaging and spectral analysis of primary inputs used in CEA could enable validation of the quality and authenticity of them. Furthermore, these tools can also enable the evaluation of these primary inputs during storage and handling. Developing ML/AI tools based on predictive relationships between the imaging or spectral features of these primary inputs and microbial analysis could provide a potential surrogate marker for assessing microbial contamination (Qian, Murphy, et al., 2023).

Water, as a critical primary input and route of widespread contamination, is an important point for microbial monitoring (Sela Saldinger et al., 2023). Microbial contamination of water can be addressed by developing a robust analysis of water quality and treatment of water. AI approaches can aid in automating the analysis of water quality, and develop predictive models of water quality for different seasons based on the geographical location and local water sources used for CEA operations (Gao et al., 2023; Ojo & Zahid, 2022; Ullo & Sinha, 2020). In addition, AI approaches could also be developed to improve and evaluate the efficacy of water treatment technologies.

CHALLENGES FOR AI TOOL APPLICATIONS TO FOOD SAFETY IN CEA

While there is enthusiasm for the use of digital and AI tools to improve food safety (e.g., the US FDA's "Smarter Food Safety Initiative" (Hamilton et al., 2023), there are limited examples of successful practical applications of these tools to CEA food safety. Due to the relative newness of the CEA sector, a specific challenge faced by many firms is a lack of foundational food safety plans and systems (Hamilton et al., 2023), which poses a major challenge for the application of AI for food safety. For example, many firms have not

implemented stringent (or any) cleaning and sanitation breaks between growing lots and reuse substrates and water without treatments (Dong & Feng, 2022). Furthermore, more automated systems that utilize moving trays with pulleys and motors (i) are rarely cleaned/sanitized and (ii) are manufactured in a way that makes equipment challenging to effectively clean (i.e., filled with hard to reach nooks and crannies, impossible to reach spots, difficult to take apart and put together) (Hamilton et al., 2023). Lack of separation between growing and processing, and inadequate food safety knowledge commonly seen among startups and newer food industries also may represent a foundational challenge for some facilities. For facilities at earlier stages of their food safety maturity, it may not be appropriate to implement high tech data intensive AI strategies until appropriate foundational food safety practices are in place, including basic food safety training programs, which can be highly effective at reducing the risk of microbial contamination (Ilic et al., 2017). Additionally, creating AI models based on data gathered from initial CEA production systems that are lacking food safety foundations may create an inappropriate starting point for the modelling and AI-based data analytics efforts, as AI models would need to be fed new data and potentially reprogrammed to accommodate the changes that occurred after implementation of foundational food safety practices. However, even in the early stages of food safety system development, CEA may benefit from using existing pre-trained Large Language Models (LLMs), such as ChatGPT or Gemini, to support basic food safety tasks, such as personnel training and development of SOPs. While these models can streamline the tasks, expert reviews are recommended to authenticate the generated information.

Food safety data availability and quality are important factors for a well-trained and validated AI tool for food safety hazards management. In order to acquire the mass of data needed, a large amount of high-quality data is required to ensure AI's reliability; the only

efficient pathway to this may often include data sharing between firms. This poses the challenge of data privacy hesitations. Food safety data are highly sensitive, due to fears of data abuse, bad publicity, reputation, liability, and the need to keep certain data (e.g., human illness data) confidential (Alexander et al., 2023; Qian, Liu, et al., 2023). CEA and other food companies can increasingly recognize the value of data sharing based on successes in other industries (from medicine to hospitality industries), where sharing of data has allowed scaling of AI applications and learning through peer networks (Qian, Liu, et al., 2023). Improved data sharing, including the use of shared data in AI models, has the potential to (i) provide food safety benchmarks for the industry and (ii) facilitate better business and food safety decisions. Thus, there is a need for research geared toward a better understanding of data sharing obstacles and the development of data infrastructure and algorithms that secure the privacy of users who engage in data sharing. One way of addressing this challenge is through federated Learning (FL), which has gained attention in several domains (Beyan et al., 2020; Dayan et al., 2021; Deist et al., 2020; Gavai et al., 2023; van Soest et al., 2018). In a federated environment, data remains secure within the physical location (i.e., data station) of its owners. Instead of transferring data, the model moves between these locations, effectively updating the model parameters from the data at the respective data stations, abiding by privacy principles. For example, Gavai et al. (Gavai et al., 2023) developed a Federated Bayesian Network (BN) model to predict food fraud, which demonstrated the applicability of the federated BN in food fraud; they anticipated that such a framework may support stakeholders in the food supply chain for better decision-making regarding food safety control while still preserving the privacy and confidentiality nature of these data. In addition to the amount of data needed, the quality of data is also imperative. To preserve data quality, monitoring data needs to be consistent and standardized throughout the data

collection period and even beyond the project time to enable a sustainable data source for further model improvement and validation. This can be challenging to apply from firm to firm as each company will have different data collection methods, data labeling, and different streams of data. This limits AI's applicability to the sector. While there is a growing trend of high-tech precision farms, there still remains a number of firms that use traditional produce growing and data monitoring methods such as pen and paper data collection. Some firms simply lack monitoring of growing variables altogether. A unique aspect of food safety data, especially microbial concentration data, is that contamination, while serious, is often rare. Contamination with pathogens would be typically detected in only a few samples, and the majority of the samples would be non-contaminated or contaminated below detection limits. These unbalanced datasets need to be handled carefully in the modelling process to reach a desired prediction accuracy for the positive samples.

It is important to realize the need to develop human resources in parallel with the development of technologies for improving the food safety of CEA-grown produce. This emphasizes the need for cross-disciplinary training between domain knowledge and data science/engineering disciplines. Additionally, to be able to leverage the full potential of AI technologies in CEA food safety, these technologies will need to present users with interpretable and useful information through an effective human-machine interface. Not only will users need to interpret results, but they will need to understand the models to address concerns about false positives and business liability as well.

Finally, to drive appropriate adoption of AI, economic aspects and costs and benefits of adopting AI systems may need to be quantified, including to understand opportunity costs that may be associated with implementing AI to help assure food safety (as there may be other food

safety investments that generate a greater risk reduction in return for a given investment). Food safety is a unique business aspect because, while required, it is often seen as a cost center; a reduction in food safety risk rarely leads to a quantifiable direct increase in profits, but rather a reduction in potential capital lost through a food safety incidence (e.g., a recall). Research geared towards identifying a connection between reduction in food safety risk or recall risk and profits may help with decision making on AI implementation and incentivize companies to invest in shifting their food safety strategy towards AI and digital tools. At the same time, changes in the legal environment are needed to alleviate the barriers to adopting these technologies, for example to alleviate industry concerns about the potential increase in the liability and expense from using food safety predictions and knowledge generated by AI and digital tools (Alexander et al., 2023).

CONCLUSIONS

While it is enticing to conclude that AI for microbial food safety is a wise application for CEA due to the industry's reputation as being "high-tech," there are still many challenges that need to be overcome prior to the adoption of this technology. Namely, there is an effort needed from academia and industry to (i) research and better understand the contamination sources, routes and microbial environment of known and unknown pathogens in a variety of CEA facilities, (ii) implement stronger, basic facility hygiene and sanitation practices and (iii) generate vast amounts of high quality data sources. Additionally, the creation of AI models needs to take into account the wide range of CEA facilities and data infrastructures and, through this, the vast amounts of private data required to be shared. While there are opportunities (such as the use of federated learning) to circumvent data privacy, the large majority of private firms are still reluctant to share confidential data. The range of CEA facilities not only makes it challenging to have one model fit multiple firms but it also poses the challenges of (i) data from different

systems “being able to talk to each other” and (ii) application of AI models into systems with ranges of technological maturity. AI for microbial food safety in CEA is most likely utilized best towards specific food safety related challenges, such as validating clean breaks between lots, and allowing for targeted and specific microbial sampling plans to capture rare but serious contamination instances. Finally, it is important to remember that while AI can be seen as an attractive “fits all solution”, food safety risks carry numerous interdisciplinary and intricate consequences for both firms and consumers, and ultimate decisions regarding such risks should be made by humans.

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CHAPTER III

NON-SPOREFORMING THERMODURIC BACTERIA VARY CONSIDERABLY IN THERMAL RESISTANCE IN MILK MEDIA BETWEEN STRAINS FROM THE SAME GENUS*

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ABSTRACT

Thermoduric bacteria, defined as those that survive temperatures considerably above their maximum growth temperature, are enumerated in milk using the laboratory pasteurization count test. This test was originally developed to detect insufficient hygiene in on-farm milking equipment but also consequently selects for bacteria that show limited cell count reductions under vat pasteurization conditions which utilizes the same time and temperature treatment as the laboratory pasteurization count test (i.e., 63°C for 30 minutes). Contemporary studies that have examined thermoduric bacterial populations in raw milk supplies are concentrated primarily on sporeforming bacteria, which have a distinct mechanism of thermal resistance compared to non-sporeforming thermoduric bacteria, namely the production of endospores. While sporeforming bacteria are of particular importance because of their thermal resistance and their ability to cause quality defects in dairy products following processing, non-sporeforming thermoduric bacteria can also cause a range of quality defects in dairy products like fluid milk, cheese, and yogurt. Non-sporeforming thermoduric bacteria are reported to enter the dairy supply chain through various points at the farm and processing facility, which can complicate troubleshooting and tracking efforts when these organisms are implicated in finished product quality defects. While there is no regulatory limit for thermoduric bacteria in raw milk, the Laboratory Pasteurization Count currently serves as an industry relevant raw milk quality test. However, this test has some limitations, the primary being that the test selects for a broad group of thermoduric bacteria, many of which are not reported to survive high-temperature, short-time pasteurization, the most commonly used pasteurization method in the United States. Therefore, the goals of this study were to i) assemble a set of non-sporeforming thermoduric bacteria previously isolated from heat treated dairy matrices, and ii) use that set to identify one or more practical laboratory

enumeration method(s) that could be used to select for groups of non-sporeforming thermoduric bacteria that exhibit limited cell count reductions when subjected to a heat treatment more similar to high-temperature, short-time pasteurization. To that end, a set of 38 isolates of non-sporeforming thermoduric bacteria isolated from naturally contaminated heat treated dairy matrices representing nine genera (*Brachybacterium, Corynebacterium, Enterococcus, Kocuria, Macrococcus, Microbacterium, Micrococcus, Staphylococcus,* and *Streptococcus*) were assembled for further examination. Isolates representing these nine genera were inoculated into skim milk broth, and independently subjected to four different heat treatments (A: 63°C for 30 minutes, B: 65°C for 15 minutes, C: 68°C for 7 minutes and D: 70°C for 5 minutes), followed by plating using two different media types (Standard Methods Agar and Aerobic Count Petrifilms), each of which were incubated and enumerated after three different incubation periods (24h, 48h, and 72h) at 32°C. This scheme resulted in 24 unique method combinations applied to each isolate tested. Results from each unique method were compared to the gold standard method (i.e., Laboratory Pasteurization Count test; 63°C heat treatment for 30 minutes, pour plating using Standard Methods Agar, followed by incubation for 48h at 32°C). All isolates from *Corynebacterium, Staphylococcus,* and *Macrococcus* exhibited >5 log CFU/mL cell count reductions post-heat treatment regardless of heat treatment, media, or incubation time, and therefore they were excluded from most analyses. Results for the remaining six genera indicate that media type was associated with considerable genus-specific differences, with Aerobic Count Petrifilms over estimating cell count reductions (i.e., growth was limited on Petrifilms compared to Standard Methods Agar) for *Kocuria, Microbacterium,* and *Streptococcus* suggesting that Aerobic Count Petrifilms may not be an appropriate media for enumerating these genera. Heat treatment D (i.e., 70°C for 5 minutes) showed a trend for higher reduction in cell counts

compared to heat treatment A (i.e., 63°C for 30 minutes; i.e., gold standard) although the difference did not reach the level of statistical significance. Our results also suggest that there is considerable variability in thermal resistance between isolates of the same genus, with some isolates within the same genus showing up to a 5 log CFU/mL difference in cell count reductions regardless of heat treatment. These phenotypic differences may be driven by yet unknown genotypic features among thermoduric bacteria that should be further examined. This study provides an update to the existing knowledge of non-sporeforming thermoduric bacteria and future studies should focus on evaluating non-sporeforming thermoduric bacteria in naturally contaminated dairy products.

INTRODUCTION

The standard method used for enumerating total thermoduric bacteria, those which can survive temperatures significantly above their maximum growth temperature (Boor and Martin, 2024), in raw milk is the Laboratory Pasteurization Count (**LPC**), which involves heat treating raw milk to 63°C for 30 minutes, followed by pour plating on Standard Methods Agar (**SMA**), and incubation for 48h at 32°C (Boor and Martin, 2024). A limitation of the LPC method is that it selects for a broad group of thermoduric bacteria (Lee et al., 2024) that do not align well with the populations found after the most commonly utilized heat treatments in the dairy industry (i.e., High Temperature Short Time [**HTST**] pasteurization). In the dairy continuum, there are two primary groups of thermoduric bacteria,, including sporeforming bacteria, such as *Paenibacillus* and *Bacillus* (Ivy et al., 2012; Buehner et al., 2014; Ribeiro Júnior et al., 2018), and non-sporeforming thermoduric bacteria (**NSTB**), such as *Brachybacterium, Kocuria*, and *Enterococcus* (Ribeiro Júnior et al., 2018; Lee et al., 2024). Both groups can survive commonly

applied pasteurization and processing hurdles in the dairy industry and cause quality-related defects (Washam et al., 1977; Ribeiro Júnior et al., 2018; Mnif et al., 2020). However, there are key differences between these groups that have practical implications for dairy industry stakeholders such as i) mechanisms of thermal resistance, ii) extent of thermal resistance, and iii) primary sources. Sporeforming bacteria have the unique ability to form endospores (i.e., spores), which can survive harsh environments (e.g., heat stress, low water activity) while remaining metabolically inactive and then can return to a vegetative state once environmental conditions become favorable again (Doyle et al., 2015). This mechanism of thermal resistance allows sporeformers, in their spore form, to survive considerably higher temperatures than NSTB (Besten et al., 2018; Lindsay et al., 2021). Sporeformers in their spore form, are the primary group surviving HTST pasteurization (Ranieri and Boor, 2009) (i.e., heat treatment of minimum 72°C for 15 seconds) which only a small subgroup of NSTB survive (Washam et al., 1977; Ranieri et al., 2009). Research on thermal mechanisms and thermal resistances of sporeforming bacteria in dairy is well established (Besten et al., 2018), while research regarding thermal resistance mechanisms and thermal resistances of NSTB is largely lacking. Some mechanisms of thermal resistance for NSTB are likely driven primarily by genotypic features (Besten et al., 2018). For example, a high DNA guanine (G) and cytosine (C) content (such as that is found in NSTB genera like *Brachybacterium, Corynebacterium,* and *Micrococcus* [Ohama et al., 1990; Ikeda and Nakagawa, 2003; Bellassi et al., 2020]) protects against DNA denaturation and damage during exposure to heat due to the strength of the GC triple hydrogen (Weissman et al., 2019; Hu et al., 2022). Other genetic mechanisms that may drive NSTB thermal resistance are a cell's ability to repair heat damage and the presence of genes or mutations that encode for regulators for heat stress response (Besten et al., 2018). Lastly, these two groups of thermoduric bacteria

(i.e., sporeformers and NSTB) are associated with different primary sources regarding their introduction into raw milk. In particular, sporeformers are ubiquitous in natural environments, particularly soil or decaying plant material (Martin et al., 2023b). Their point of entry into the dairy supply continuum is at the milk harvest step (Scheldeman et al., 2005; Martin et al., 2019). Specific research regarding sources of NSTB in raw milk, on the other hand, is lacking. However, historically, thermoduric bacteria have been associated with unhygienic milking equipment/storage (Byrne and Bishop, 1991; Martin et al., 2023a). For example, Elmoslemany et. al (2010) found that high levels of thermoduric bacteria were associated with specific on-farm practices including lack of a water purification system, on-farm plate cooler (which are challenging to properly clean and sanitize), and inadequate frequency of an acid wash. These distinct sources of thermoduric bacteria, along with the differences in mechanisms of thermal resistance, highlight the limitations of the currently used LPC method

The dairy industry is in need of a test that enumerates sporeforming bacteria, as well as NSTB that can survive HTST pasteurization. To that end, the goals of this study were to i) identify a representative set of NSTB previously isolated from heat treated dairy products, and ii) utilize these isolates to identify a practical laboratory method(s) that can be used by members of the dairy industry (e.g., processors, cooperatives, farmers) to select for a sub group of NSTB based on thermal resistance. The results from this study can be used as a starting point for further research regarding NSTB in naturally contaminated dairy products.

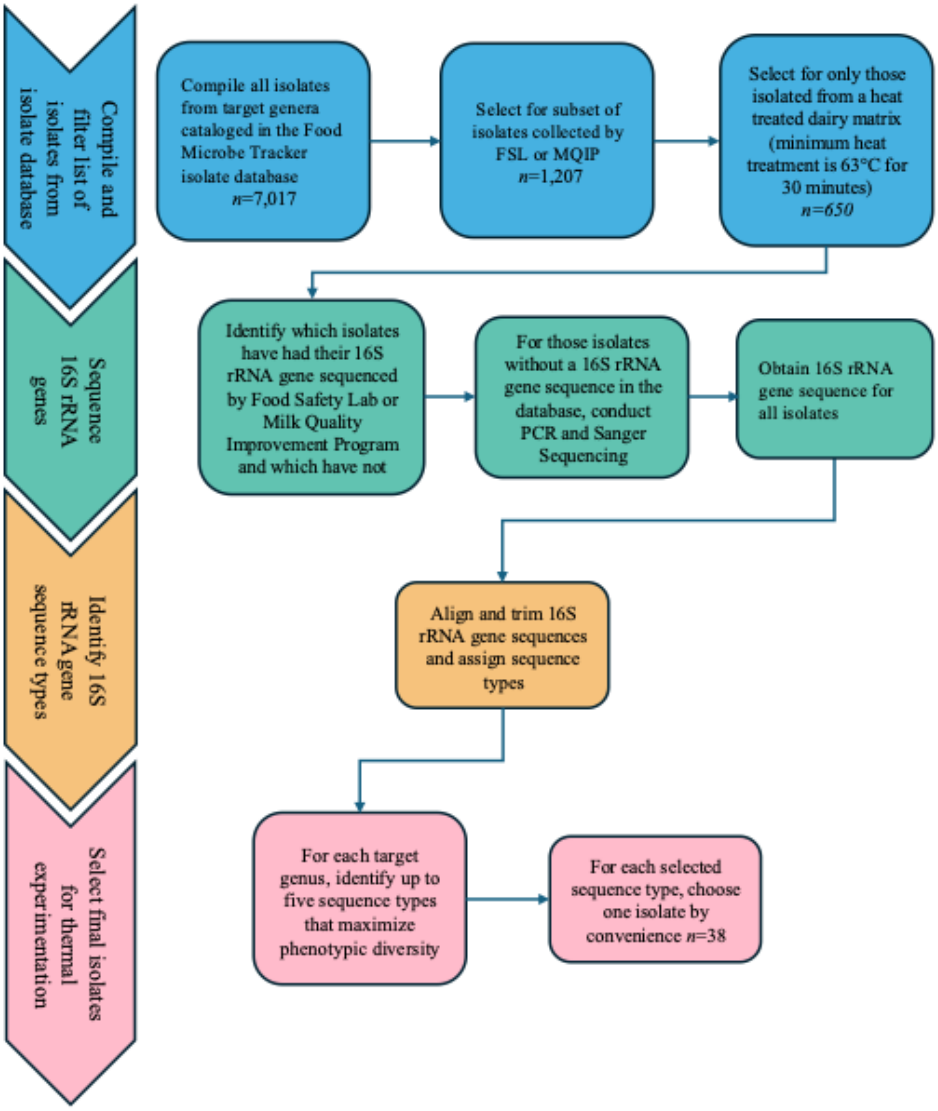
MATERIALS AND METHODS

Identification and selection of bacterial representatives of NSTB

A previous comprehensive study of lab pasteurized organic raw milk (Lee et al., 2024) evaluated LPCs in organic raw milk samples from 94 farms across the US, and identified eight

non-sporeforming thermoduric genera, including *Brachybacterium, Kocuria, Microbacterium, Micrococcus, Enterococcus, Staphylococcus, Streptococcus,* and *Corynebacterium.* Further review of the available literature regarding NSTB indicated that *Macrococcus* has also previously been identified as a thermoduric genus isolated from dairy (Ribeiro Júnior et al., 2018; Sanschagrin et al., 2024), and therefore this genus was also included in our study. Isolates from the target nine genera were compiled (*n*=7,017) from the Food Safety Laboratory (**FSL**) and Milk Quality Improvement Program (**MQIP**) bacterial isolate collection (https://www.foodmicrobetracker.net/login/login.aspx) which consists of over 100,000 bacterial isolates collected from a variety of food and non-food environments. The list of isolates was further narrowed to select only for isolates; i) collected by FSL or MQIP (*n*=1,207), and ii) isolates originating from dairy matrices subjected to a minimum heat treatment of 63°C for 30 minutes (i.e., LPC treatment or vat pasteurization) (*n*=650) (Figure 3.1).

Figure 3.1. Flow chart outlining method for the generation of a set of non-sporeforming thermoduric bacterial isolates from Cornell University's bacterial isolate collection (https://www.foodmicrobetracker.net/login/intro.aspx) for evaluation of thermal resistance under various modified laboratory heat treatment, media and incubation parameters. Target genera included *Brachybacterium, Corynebacterium, Enterococcus, Kocuria, Macrococcus, Microbacterium, Micrococcus, Staphylococcus,* and *Streptococcus*. FSL stands for Food Safety Laboratory and MQIP stands for Milk Quality Improvement Program



Isolates without 16S rRNA gene sequencing data were subjected to PCR and Sanger sequencing of the 16S rRNA gene as previously described by Lee et. al (2024) using the forward primer 16S-PEU7 (Rothman et al., 2002) and the reverse primer16S-DG74 (Greisen et al., 1994). Once all 16S rRNA gene sequences for each of the 650 isolates were obtained, sequences were aligned in MEGA version 11.0.13 (Tamura et al., 2021). Sequences were trimmed and compared to identify unique sequence types (**ST**). To capture the phenotypic diversity (i.e., maximizing single nucleotide polymorphism [**SNP**] differences between STs) of the nine genera studied here, a maximum of five STs were selected per genus. For genera that had <5 total STs (i.e., *Macrococcus* and *Corynebacterium*), all available STs were selected. Furthermore, for cases where all STs were within 2 SNPs of each other (i.e., *Brachybacterium*), STs were selected using a random number generator. Once all STs were selected, 1 isolate from each ST was conveniently selected to form a final NSTB set (*n*=38) for further characterization (Table 3.1).

Table 3.1 Characteristics of isolates selected for experimental assessment of thermal resistance including genus, isolate, 16S rRNA gene sequence type, isolate source, and reference (if available)

Isolate[1]	16S rRNA Gene Sequence Type	Source[2]	Reference[3]
Brachybacterium (*n*=5)			
FSL E3-2387	51	Heat treated organic raw milk	NA
FSL E3-2184	52	Heat treated raw milk	NA
FSL E3-2762	54	Heat treated organic raw milk	NA
FSL E3-2186	47	Heat treated raw milk	NA
FSL E3-2582	56	Heat treated organic raw milk	NA
Corynebacterium (*n*=3)[4]			
FSL W6-0345	59	Heat treated raw milk	NA
FSL R7-0098	58	Pasteurized 2% fat milk	NA
FSL K6-0767	57	Pasteurized cheese whey	(Kent et. al, 2015)
Enterococcus (*n*=5)			
FSL R5-0363	4	Heat treated 2% fat milk	(Ranieri and Boor, 2009)
FSL E2-0535	1	Ultra filtrated pasteurized milk	NA
FSL W6-0423	13	Heat treated raw milk	(Trmčić et al., 2015)
FSL R10-0012	11	Pasteurized skim chocolate milk	NA
FSL E3-3477	9	Heat treated raw milk	NA
Kocuria (*n*=4)			
FSL K6-0771	66	Whey protein concentrate	(Kent et. al, 2015)

FSL W5-0894	63	Pasteurized milk	NA
FSL E3-2311	49	Heat treated raw milk	NA
FSL A6-305	60	Heat treated raw milk	NA
Macrococcus (*n*=2)[4]			
FSL L6-0038	15	Heat treated raw milk	NA
FSL C4-0051	14	Pasteurized homogenized milk	NA
Microbacterium (*n*=5)			
FSL F4-0109	19	Pasteurized 2% fat milk	NA
FSL L6-0278	23	Pasteurized whole milk	NA
FSL R11-0218	29	Pasteurized cream-line milk	NA
FSL F4-0288	21	Pasteurized 2% fat milk	NA
FSL W6-0342	105	Heat treated raw milk	NA
Micrococcus (*n*=5)			
FSL E3-2576	73	Heat treated organic raw milk	NA
FSL M7-0406	74	Heat treated raw milk	NA
FSL W6-0814	80	Pasteurized 2% milk	NA
FSL E2-4746	71	Heat treated raw milk	NA
FSL M7-0630	75	Heat treated raw milk	NA
Staphylococcus (*n*=4)			
FSL R5-0191	88	Pasteurized 2% fat milk	(Ranieri and Boor, 2009)
FSL R5-0454	89	Pasteurized 2% fat milk	(Ranieri and Boor, 2009)
FSL R5-0556	90	Pasteurized 2% fat milk	(Ranieri and Boor, 2009)
FSL E3-3121	83	Heat treated raw milk	NA

Streptococcus (*n*=5)			
FSL W6-0451	46	Heat treated raw milk	NA
FSL E3-3422	35	Heat treated organic raw milk	NA
FSL W6-0436	42	Heat treated raw milk	NA
FSL E3-2728	32	Heat treated organic raw milk	NA
FSL A6-307	30	Heat treated raw milk	NA

[1] FSL refers to Food Safety Laboratory.
[2] Represents the original source of the isolate. Heat treated: isolate was isolated from raw milk that had been heat treated to at least 63°C for 30 minutes; Pasteurized: isolate isolated from a product or ingredient that was pasteurized at a minimum of 63°C for 30 minutes (i.e., vat pasteurization). See Food Microbe Tracker (https://www.foodmicrobetracker.net/login/login.aspx) for more details.
[3] Indicates if isolate appears in a previous publication. NA: Not Available
[4] Fewer than 5 sequence types total were available for selection from this genus in the Food Microbe Tracker bacterial isolate collection.

Cell preparation and determination of bacterial cell concentrations

An estimate of the bacterial cell concentration of overnight cultures for each isolate in the NTSB set was determined in order to calculate inoculum concentrations to be used in the subsequent heat treatment experiments. Overnight cultures were prepared by streaking from frozen 15% glycerol stocks stored at -80°C onto pre-poured Brain Heart Infusion (**BHI**) agar plates and subsequently incubated at 32°C for 24h. Following incubation, a single colony from each BHI plate was inoculated into 5 mL of BHI broth, vortexed, and incubated at 32°C for 24h. Following the second incubation, the culture was vortexed and 1 mL was transferred onto pre-poured BHI agar plates and spread with a sterile swab to form a lawn. Plates were subsequently incubated at 32°C for 18h.

Following incubation of lawn plates, 5 mL of Phosphate Buffered Saline (**PBS**) was added to each bacterial lawn plate. Bacterial lawns were gently scraped off the agar plate with a sterile L-shaped spreader and mixed with the PBS solution. The bacterial solution was then pipetted into sterile 15 mL conical tubes and tubes were centrifuged at 2,000 x g for 20 minutes, as adapted from Glass and Doyle (1989). Following centrifugation, pellets were suspended in 5 mL of PBS and vortexed until homogenous.

The volume of bacterial cell solution used for further inoculation for heat treatment experiments was based on estimates of the initial cell concentration in the resuspended pellet which was determined in one of two ways; i) by using OD_{600} curves, or ii) by enumerating the previously mentioned bacterial lawns by pour plating with SMA tempered to approximately 45°C, followed by incubation for 48h at 32°C to achieve a known initial culture concentration. The OD_{600} curve method was not used for all isolates because this method was found to be highly variable between the expected bacterial cell concentration and the outcome. Out of the 38 total isolates, we exclusively utilized the OD_{600} curve method for only nine isolates (i.e., FSL W6-0345, FSL L6-0038, FSL F4-0288, FSL W6-0342, FSL E3-2576, FSL E2-4746, FSL R5-0556, FSL W6-0451, and FSL W6-0436), we utilized both methods for different heat treatments of four isolates (i.e., FSL A6-305, FSL E2-0535, FSL R5-0363, and FSL R7-0098), and for the remaining 25 isolates we exclusively utilized the lawn enumeration method. We do not anticipate that this change in methodology affected our results as, in all cases, actual inoculum concentrations were measured prior to heat treatment to confirm initial concentration.

Differential method for NSTB testing and microbial analysis

Each bacterial isolate was inoculated into 35 mL of sterile skim milk broth (**SMB**) in sterile 100 mL screw cap glass bottles to achieve a target inoculum concentration of

approximately 10^6 CFU/mL, as outlined above. Inoculated SMB was shaken 7 times in 25 seconds (McMahon and Headley, 2024) to ensure thorough mixing, then a 7 mL aliquot was transferred into each of four separate sterile glass 10 mL screw cap vials, one vial for each heat treatment (A-D; Table 3.2).

Table 3.2. Parameters of 24 possible testing combinations where each isolate received 4 distinct heat treatments, was plated with 2 types of media, and bacterial colonies were enumerated at all three incubation times

Method Parameters		
Heat Treatment	Media Type	Incubation Time at 32°C
63°C for 30 minutes (A)[1]	Standard Methods Agar[1]	24h
65°C for 15 minutes (B)	Aerobic Count Petrifilms	48h[1]
68°C for 7 minutes (C)		72h
70°C for 5 minutes (D)		

[1] Gold standard method for reference (Boor and Martin, 2024)

An uninoculated (i.e., sterile) SMB temperature control was prepared in the same manner for each of the heat treatments. Prior to heat treatment, initial bacterial concentration was determined by pour plating 1 mL of appropriate dilutions of inoculated SMB in SMA tempered to approximately 45°C. Plates were subsequently incubated at 32°C for 48h. The order of heat treatments (A-D) was randomized on each experimental day. Vials were placed into a pre-heated water bath at each of the four treatment temperatures so that the water level exceeded the SMB level in the vial by approximately 1 cm. Following heat treatments, vials were subsequently placed into an ice bath. Once the temperature of the samples reached <6°C, samples were serially diluted and plated in duplicate onto either i) empty Petri dishes for pour plating using molten SMA tempered to approximately 45°C or ii) onto 3M™ Aerobic Count Petrifilms™ (i.e., **AC**

Petrifilms) (Neogen, Lansing, MI). To ensure the enumeration of any residual cells following heat treatments, we plated all serial dilutions between 0 (i.e., undiluted sample) and -6. All plates were incubated at 32°C, and enumerated at each incubation point (i.e., 24h, 48h, and 72h).

All AC Petrifilms were counted by hand while SMA plates were enumerated using an automated counter (Q-count, Advanced Instruments, Norwood, MA). Counts for technical replicates of plates within the appropriate countable range for each media were averaged, and then multiplied by the respective dilution factor to achieve a final bacterial cell count for each sample, expressed as log CFU/mL.

Data management and statistical analysis

Data were stored in Microsoft Access database and Microsoft Excel (Microsoft Corp., Redmond, WA). All bacterial concentration data was log transformed prior to analyses. In the occurrence of a full thermal inactivation (i.e., no growth post heat treatment), the bacterial cell count reduction was assumed to be at least the same as the starting inoculum concentration. For example, for an isolate with a starting inoculum concentration of 6.20 log CFU/mL, that exhibited no growth after the heat treatment, the cell count reduction was recorded as >6.20 log CFU/mL. Project data and code can be found at https://github.com/FSL-MQIP/Thermodurics.git. All statistical analyses were performed in R (Team, 2014). Plots were designed using ggplot2 (Wickham, 2016) or emmeans (Lenth, 2023).

Mixed linear regression models were fitted to identify important relationships between fixed variables (i.e., heat treatment, media, genera, and incubation time) and random effects (i.e., isolate and plate). "Plate" refers to either the AC Petrifilm or petri dish used to enumerated the samples for a specific isolate whereby results from the three incubation times (i.e., 24h, 48h, or

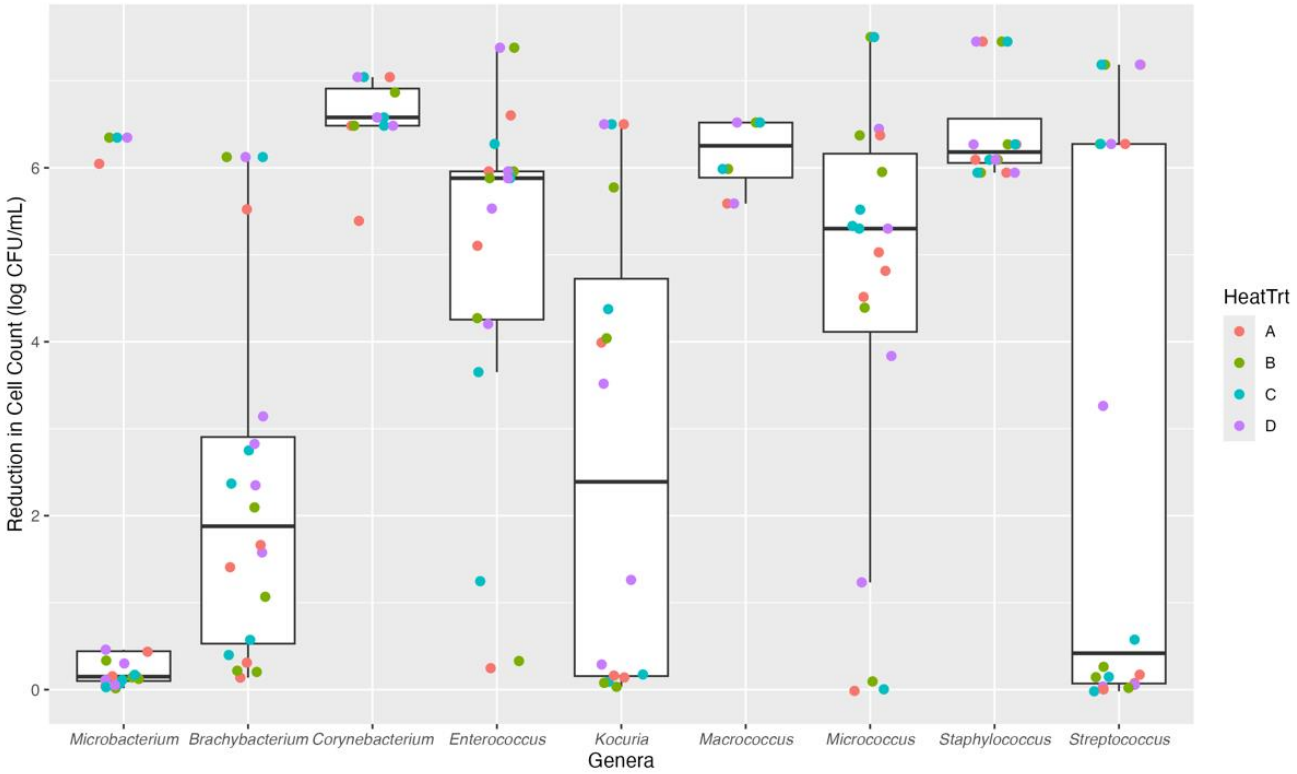
72h) are connected to a single plate. The dependent variable is cell count reduction log CFU/mL and models were created using R packages lme4 (Bates et al., 2015) and lmertest (Kuznetsova et al., 2017). Model 1 consisted of all data observations (*n*=912) while the data set used for model 2 consisted of a subset where i) *Staphylococcus, Macrococcus,* and *Corynebacterium* were removed due to our observation that all isolates demonstrated >5 log CFU/mL cell count reduction for all of the tested scenarios and ii) only 48h and 72h time points were included due to the overall limited bacterial recovery observed for most isolates at the 24h enumeration time point. This resulted in a final set of 464 data points for model 2. Both models use a reference of heat treatment A, SMA media, and a 48h incubation (i.e., gold standard) (Boor and Martin, 2024), as well as *Microbacterium* as a reference genus. Finally, an Analysis of Variance (ANOVA) was conducted for each model to identify statistically significant variables and possible interactions between variables. Estimated mean pairwise comparisons were then used to determine the key levels driving the statistical significance. An alpha level of α=0.05 was used for analysis.

RESULTS

Thermal resistance varies considerably from strain to strain within NSTB genera

Following heat treatments, reduction in bacterial cell count varied widely between genera and from isolate to isolate for many genera tested (Figure 3.2).

Figure 3.2. Box and whisker plot of cell count reductions (log CFU/mL) for isolates (*n*=38) subjected to four different heat treatments (HeatTrt) (A: 63°C for 30 minutes; B: 65°C for 15 minutes; C: 68°C for 7 minutes; D: 70°C for 5 minutes) plated with Standard Methods Agar and incubated for 48h by genus and heat treatment. The interquartile range (IQR) is represented by the top (75^{th} percentile) and bottom of the box (25^{th} percentile). The horizontal line within the box represents the median. The whiskers illustrate variability and the length of the whiskers is 1.5 times the IQR. The outliers, which do not fall within 1.5 times the IQR are shown by dots.



Using the media and incubation time from the gold standard method (i.e., SMA incubated for 48h), genera can be grouped into three distinct groups based on cell count reduction across the four heat treatment parameters. Group 1 (*n*=3) exhibited consistency in thermal resistance between all isolates tested and all heat treatments. Group 1 included *Corynebacterium*, *Micrococcus*, and *Staphylococcus*, where all isolates exhibited cell count reductions >5 log CFU/mL regardless of testing parameters. In other words, all isolates from these genera exhibited very minimal to no growth post heat treatment regardless of heat treatment, media type, or incubation time (Table 3.3). Group 2 (*n*=4) represents genera where individual isolates within each genus varied in thermal resistance but were generally consistent across all heat treatments. Group 2 includes *Microbacterium, Kocuria, Enterococcus*, and *Micrococcus.* For example, one isolate of *Kocuria* (FSL K6-0771) exhibited >5 log CFU/mL cell count reduction for every heat treatment, another *Kocuria* isolate (FSL W5-0894) exhibited a partial reduction (3.52-4.37 log CFU/mL) at every heat treatment, and the final two *Kocuria* isolates (FSL E3-2311 and FSL A6-305) exhibited a minimal cell count reduction (0.03-1.26 log CFU/mL reduction) at every heat treatment (Table 3.3).

Table 3.3 Reductions in cell count (log CFU/mL) for all isolates and heat treatments plated with Standard Methods Agar and enumerated after 48h incubation

Isolate	Heat Treatment[1]			
	A	B	C	D
Brachybacterium				
FSL E3-2387	0.14	0.20	2.37	3.14
FSL E3-2184	0.31	0.22	0.40	1.57
FSL E3-2762	1.66	1.07	0.57	2.35
FSL E3-2186	5.52	>6.12	>6.12	>6.12
FSL E3-2582	1.40	2.09	2.75	2.83
Corynebacterium				
FSL W6-0345	>7.04	6.86	>7.04	>7.04
FSL R7-0098[2]	>5.39	>6.58	>6.58	>6.59
FSL K6-0767	>6.48	>6.48	>6.48	>6.48
Enterococcus				
FSL R5-0363	6.60	>7.38	6.27	>7.38
FSL E2-0535	5.10	4.27	3.65	5.53
FSL W6-0423	>5.95	>5.95	>5.95	>5.95
FSL R10-0012	>5.88	>5.88	>5.88	>5.88
FSL E3-3477	0.25	0.33	1.25	4.20
Kocuria				
FSL K6-0771[2]	>6.51	>5.77	>6.51	>6.51
FSL W5-0894	3.99	4.04	4.37	3.52

FSL E3-2311	0.14	0.03	0.09	0.29
FSL A6-305	0.16	0.07	0.17	1.26
Macrococcus				
FSL L6-0038	>6.52	>6.52	>6.52	>6.52
FSL C4-0051	5.59	>5.99	>5.99	5.59
Microbacterium				
FSL F4-0109	0.15	0.33	0.11	0.30
FSL L6-0278	0.04	0.02	0.07	0.11
FSL R11-0218	0.14	0.14	0.03	0.05
FSL F4-0288	6.04	>6.35	>6.35	>6.35
FSL W6-0342	0.44	0.12	0.17	0.46
Micrococcus				
FSL E3-2576	-0.01[3]	0.09	0.00	1.23
FSL M7-0406	4.81	5.95	5.52	>6.45
FSL W6-0814	4.51	4.39	5.30	5.30
FSL E2-4746	5.03	>7.50	>7.50	3.84
FSL M7-0630	>6.37	>6.37	5.33	LE[4]
Staphylococcus				
FSL R5-0191	>6.09	>6.09	>6.09	>6.09
FSL R5-0454	>6.27	>6.27	>6.27	>6.27
FSL R5-0556	>5.94	>5.94	>5.94	>5.94
FSL E3-3121	>7.45	>7.45	>7.45	>7.45
Streptococcus				

FSL W6-0451	>6.27	>6.27	>6.27	>6.27
FSL E3-3422	0.08	0.14	-0.02[3]	0.04
FSL W6-0436	>7.19	>7.19	>7.19	>7.19
FSL E3-2728	0.17	0.26	0.15	0.06
FSL A6-307	0.00	0.02	0.57	3.26

[1] Heat treatment A:63°C for 30 minutes; heat treatment B: 65°C for 15 minutes; heat treatment C: 68°C for 7 minutes; heat treatment D: 70°C for 5 minutes. Greater than sign (>) represents full thermal inactivation (i.e., no colony growth post heat treatment)
[2] Isolates where all cell count reductions across heat treatments show a greater than sign (>) (i.e., indicating full thermal inactivation in all heat treatments), but show different cell count reduction values between heat treatments, indicate different starting concentration values. This difference is due to heat treatments being performed on different experimental days.
[3] Negative value (-) indicates the cell concentration measured after heat treatment was greater than the starting cell concentration.
[4] Missing value due to lab error (LE).

Furthermore, regardless of heat treatment, four of five isolates of *Microbacterium* (FSL F4-0109, FSL L6-0278, FSL R11-0218, and FSL W6-0342) exhibited <0.5 log CFU/mL cell count reduction, while the remaining *Microbacterium* isolate (FSL F4-0288) displayed >5 log CFU/mL cell count reductions at all heat treatments (Table 3.3). This illustrates consistency in cell count reduction regardless of heat treatment but differences in thermal resistance between isolates of the same genus. Finally, Group 3 (n=2) represents genera where cell count reductions varied by isolate of the same genus and cell count reductions within a single isolate varied based on heat treatment. Group 3 includes *Brachybacterium* and *Streptococcus*. *Brachybacterium* exhibited a wide range of thermal resistance between isolates and heat treatments. Two isolates, FSL E3-2387 and FSL E3-2762, exhibited a range of cell count reduction across heat treatments of 0.14-3.14, and 0.57-2.35 log CFU/mL, respectively (Table 3.3). Conversely, *Brachybacterium* isolate FSL E3-2186, exhibited a range in cell count reductions of 5.52-6.12 log CFU/mL across heat

treatments, illustrating variability in thermal resistance across isolates. Similarly, three *Enterococcus* isolates (FSL W6-0423, FSL R10-0012, and FSL R5-0363) exhibited similar behavior, showing substantial cell count reductions (ranging from 5.95-7.38 log CFU/mL across all heat treatments) while another *Enterococcus* isolate (FSL E3-3477) displayed minimal cell count reductions when subjected to heat treatments A, B, and C (0.25, 0.33, and 1.24 log CFU/mL, respectively), but when subjected to treatment D, we found a 4.20 log CFU/mL reduction. Additionally, one isolate of *Enterococcus* (FSL E2-0535) showed moderate and varied cell count reductions ranging from 3.65 to 5.53 log CFU/mL across different heat treatments (Table 3.3).

Test parameters including media type and incubation time impact thermoduric bacterial recovery following heat treatment

Here, we used a mixed effects linear model to evaluate the impact of test method parameters on the resulting thermal inactivation of 38 NSTB isolates. Our results from model 1 identified significantly larger cell count reductions (i.e., less bacterial recovery) at 24 hours of incubation, compared to both 48 hours and 72 hours ($p < 0.001$ for both) (Supplemental Figure 3.1).To this point, The estimated mean pairwise comparison differences in cell count reduction between 24h and 48h and between 24h and 72h were 1.19 log CFU/mL and 1.38 log CFU/mL, respectively. However, this reflects the limited bacterial growth after 24 hours of incubation rather than a true thermal reduction. For instance, using the reference media and heat treatment (i.e., SMA and heat treatment A; i.e., gold standard), none of the *Microbacterium* isolates tested showed growth after 24h of incubation. However, by the 48h enumeration time point, four of five *Microbacterium* isolates (specifically FSL F4-0109, FSL L6-0278, FSL R11-0218, and FSL

W6-0342) had bacterial counts such that the cell count reduction was calculated to be <1 log CFU/mL from the starting inoculation concentration (Supplemental Figure 3.1). Differences in the calculated log cell count reduction for these *Microbacterium* isolates (i.e., FSL F4-0109, FSL L6-0278, FSL R11-0218, and FSL W6-0342) from the 24h time point to the 48h time point were 5.92, 5.94, 5.84 and 6.97 log CFU/mL respectively. Thus, our experiments suggest that enumeration of the NSTB evaluated here at 24h results in underestimation of true bacterial counts, and was observed across many of the genera tested (Supplemental Figure 3.1), but was not observed at 48h or 72h.

After removing the 24-hour enumeration time point from our analysis in model 2 (as well as removing genera that exhibited >5 log CFU/mL regardless of any of the tested scenarios [i.e., *Staphylococcus, Corynebacterium* and *Macrococcus*]), our results indicated that an extended incubation time of 72h resulted in significantly smaller cell count reductions compared to cell count reductions enumerated after 48h ($p<0.01$) (Table 3.4).

Table 3.4. Four way Analysis of Variance (ANOVA) summary for the mixed effect linear model using data from a subset of isolates[1] tested using a combination of heat treatments, media type, and incubation time[2]

	Four Way Analysis Of Variance Output					
	Sum[3] Sq	**Mean Sq[3]**	**NumDF[3]**	**DenDF[3]**	**F Value[3]**	**Pr(>F)[3]**
Genera	8.07	1.61	5	23.00	1.35	0.28
Heat Treatment	8.81	2.94	3	68.72	2.46	0.07
Incubation Time	8.64	8.64	1	274.36	7.24	<0.01
Media Type	84.11	84.11	1	274.36	70.51	<0.01
Genera: Heat Treatment	23.13	1.54	15	68.65	1.29	0.23
Genera: Incubation Time	9.05	1.81	5	274.31	1.52	0.18
Heat Treatment: Incubation Time	0.60	0.20	3	274.36	0.17	0.92
Genera: Media Type	70.63	14.13	5	274.31	11.84	<0.01
Heat Treatment: Media Type	0.76	0.25	3	274.36	0.21	0.89
Incubation Time: Media Type	2.50	2.50	1	274.36	2.09	0.15
Genera: Heat Treatment: Incubation Time	1.10	0.07	15	274.28	0.06	1.00
Genera: Heat Treatment: Media Type	8.01	0.53	15	274.28	0.45	0.96
Genera: Incubation Time: Media type	8.68	1.74	5	274.31	1.46	0.20
Heat Treatment: Incubation Time: Media Type	0.18	0.06	3	274.36	0.05	0.99
Genera: Heat Treatment: Incubation Time: Media Type	0.63	0.04	15	274.28	0.03	1.00

[1] Data for this model (*n*=464) represents a subset of the full data set where i) *Staphylococcus, Macrococcus,* and *Corynebacterium* were removed due to all isolates exhibiting >5 log CFU/mL cell count reductions for all of the tested scenarios and ii) only 48h and 72h time points were included.

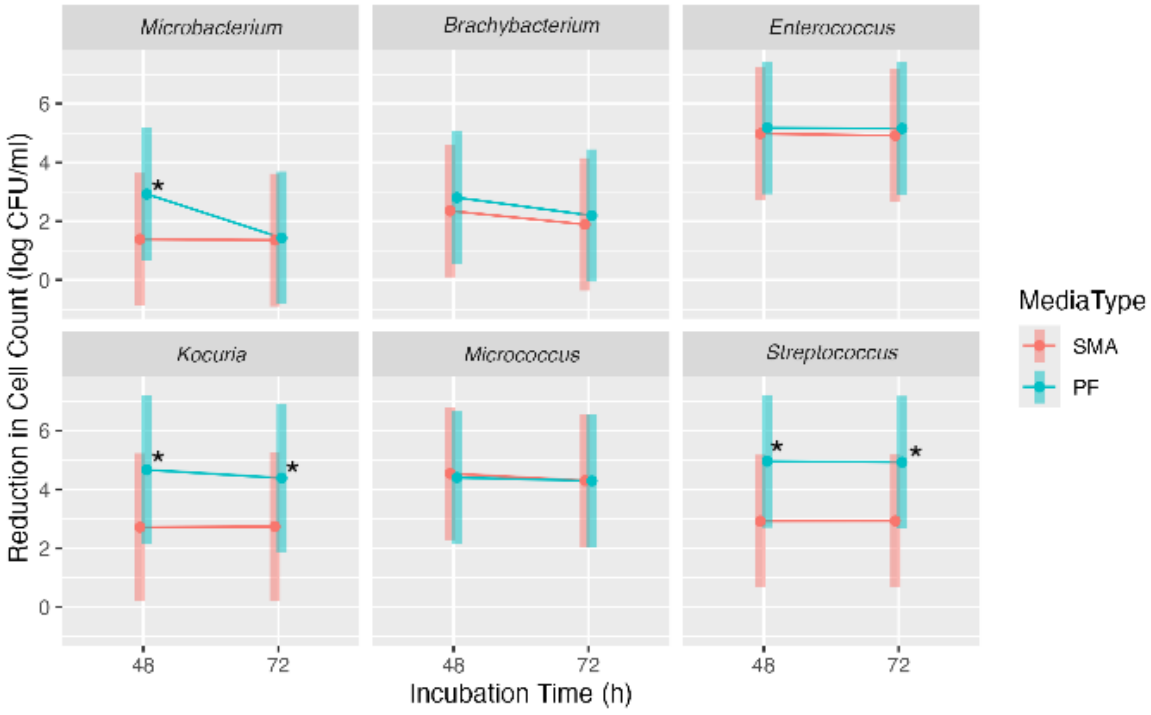
[2] Heat treatments include 63°C for 30 minutes (A), 65°C for 15 minutes (B), 68°C for 7 minutes (C), 70°C for 5 minutes (D). Media are Standard Methods Agar (SMA) and Aerobic Count Petrifilms. Incubation times include 24h, 48h, and 72h.

[3] Four way ANOVA statistics of the mixed effect linear model by reference coding (R default). Sum sq: the sum of squares due to the factor or two-way, three-way or four-way interaction; Mean sq: mean of the sum of squares due to the factor or two-way, three-way or four-way interaction; NumDF: numerator degree of freedom; DenDF: denominator degree of freedom; *F* value: F statistic; Pr(>F): the *p* value

However, the effect size was relatively small, with an estimated mean pairwise difference in cell count reductions between 48h and 72h of only 0.28 log CFU/mL. These results indicate that a 24h incubation period is insufficient for enumerating NSTB, and while the 72h incubation period was significantly associated (p<0.01) with larger recovery of NSTB compared to the 48h incubation period, the effect size was limited. Overall, based on these results, a 48h incubation period is appropriate for the recovery and enumeration of NSTB.

The ANOVA output from model 2 (Table 3.4) indicated that the media (i.e., AC Petrifilm) showed a significantly positive association (p < 2.48e-15)(Table 3.4) with cell count reduction compared to the reference (i.e., SMA). This suggests that use of AC Petrifilm resulted in seemingly larger cell count reductions than SMA. However, this actually represents lower bacterial recovery on AC Petrifilm. The estimated means pair wise comparison identified a difference of 0.86 log CFU/mL in cell count reduction between the two media. Additionally, the interaction effect between genus and media type was also significant (p<0.01) (Table 3.4), indicating that the impact of media is dependent on genera. Specifically, *Kocuria, Microbacterium,* and *Streptococcus* exhibited significantly (p<0.0001, p=0.001, and p<0.0001 respectively) larger cell count reductions with the use of AC Petrifilms compared to SMA. The estimated means pair wise comparison identified differences in calculated cell count reduction with use of AC Petrifilms of 1.80, 0.80, and 2.02 log CFU/mL for *Kocuria, Microbacterium,* and *Streptococcus,* respectively (Figure 3.3) compared with pour plating on SMA.

Figure 3.3. Estimated mean reductions in cell count (log CFU/mL) from a mixed effect linear model using a subset of data (*n*=464) where i) *Staphylococcus, Macrococcus* and *Corynebacterium* were removed due all isolates exhibiting >5 log CFU/mL cell count reductions for all of the tested scenarios and ii) only 48h and 72h time points were included, with 95% confidence intervals for each genus as indicated by the vertical lines at each incubation point, across heat treatments A-D (A: 63°C for 30 minutes; B: 65°C for 15 minutes; C: 68°C for 7 minutes; D: 70°C for 5 minutes). SMA=Standard Methods Agar and PF= Aerobic Count Petrifilms. An asterisk (*) indicates estimated means pair wise comparison identified significant (p<0.05) differences in cell count reductions between media at time point.

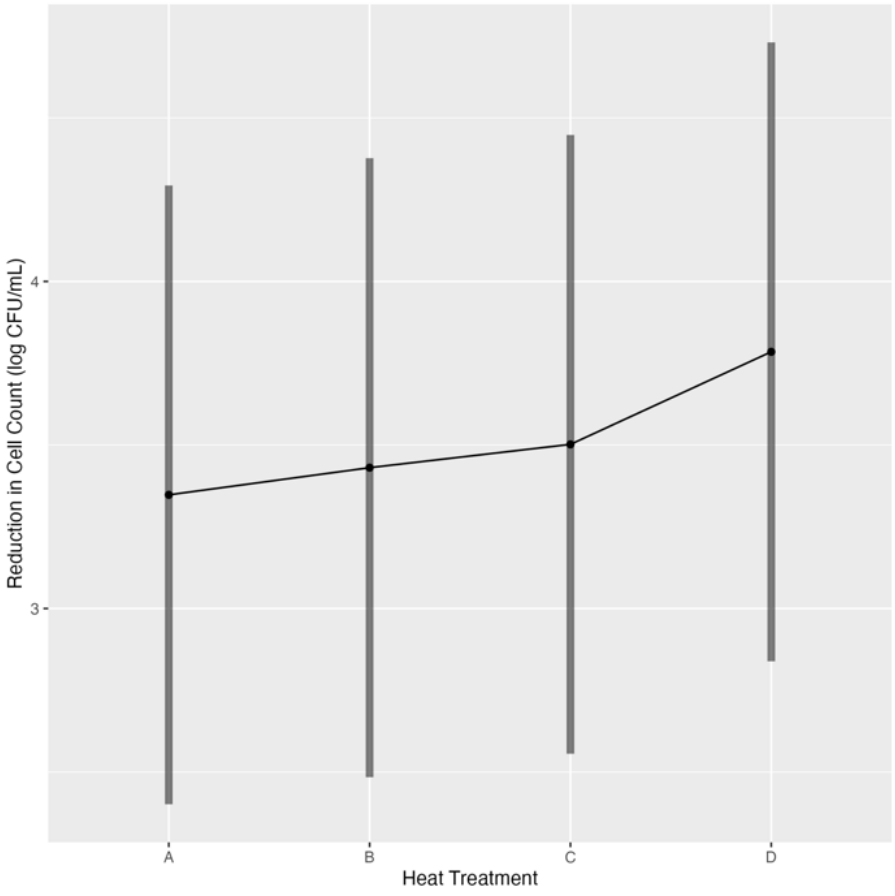


Additionally, even larger differences between media for individual isolates belonging to the above-mentioned genera were observed. For example, after 48h of incubation, two *Streptococcus* isolates, FSL E3-2728, and FSL A6-307 showed considerable differences in cell count reduction by media, with both isolates exhibiting >5 log CFU/mL difference between Petrifilm AC and pour plating with SMA, regardless of heat treatment (Supplemental Figure 3.1). The remaining genera used in model 2, *Brachybacterium, Micrococcus,* and *Enterococcus,* exhibited smaller overall cell count reduction differences between media with estimated means pair wise comparisons of 0.38, 0.07, 0.21 log CFU/mL, respectively (Figure 3.3) Overall, these results indicate that AC Petrifilm is not the ideal media to recover and enumerate NSTB.

Heat treatment of NSTB strains in SMB at 70°C resulted in a larger cell count reduction, compared to the gold standard heat treatment

The ANOVA results of model 2 (Table 3.4) identified that the variable of heat treatment showed a nearly significant (p=0.07; Figure 3.4), positive association with cell count reduction overall.

Figure 3.4. Estimated mean reductions in cell count (log CFU/mL) from a mixed effect linear model using a subset of data (*n*=464) where i) *Staphylococcus, Macrococcus* and *Corynebacterium* were removed due all isolates exhibiting >5 log CFU/mL cell count reductions for all of the tested scenarios, and ii) only 48h and 72h incubation points were included, with 95% confidence intervals for each heat treatment as indicated by the vertical lines at each heat treatment (A-D: A: 63°C for 30 minutes; B: 65°C for 15 minutes; C: 68°C for 7 minutes; D: 70°C for 5 minutes) across all genera (i.e., *Brachybacterium, Enterococcus, Kocuria, Microbacterium, Micrococcus, Streptococcus*), media (Standard Methods Agar and Aerobic Count Petrifilms), and incubation points (48h and 72h).



However, the ANOVA also identified there was no significant (p>0.05) interaction between heat treatment and genus or any other variables. Despite the lack of a significant p value from the ANOVA associated with heat treatment, a post-hoc analyses was conducted to identify which heat treatment levels were driving the nearly significant association between heat treatment and cell count reduction. Pairwise comparison of estimated means identified that heat treatment D (70°C for 5 minutes) was nearly significantly different (p=0.07) from heat treatment A (63°C for 30 minutes, i.e., the gold standard). Heat treatments B (65°C for 15 minutes) and C (68°C for 7 minutes) were not significantly (p>0.05) different from heat treatment A (i.e., gold standard) (p=0.96 and 0.80, respectively; Figure 3.4). Furthermore, pairwise comparison of estimated means identified that heat treatment D has an estimated increase in cell count reduction of 0.44 log CFU/mL more than heat treatment A while heat treatment B and heat treatment C only had minor increases in cell count reduction of 0.08 log CFU/mL and 0.15 log CFU/mL, respectively. These findings highlight the potential of heat treatment D to select for a subset of NSTB that show limited cell count reductions at temperatures closer to HTST pasteurization temperatures.

DISCUSSION

Thermoduric bacteria have been a focus of non-regulatory, quality testing programs in the US since the mid-20th century (Hileman, 1940; Byrne and Bishop, 1991; Ribeiro Júnior et al., 2018). This important group of bacteria are comprised of sporeforming and non-sporeforming bacteria that survive exposure to temperatures above their maximum growth temperatures (Boor and Martin, 2024). Non-sporeforming thermoduric bacteria can cause a range of undesirable quality related defects in dairy products (e.g., fluid milk, cheese, yogurt) such as off-flavors and decrease shelf life (Washam et al., 1977; Ribeiro Júnior et al., 2018). While there is extensive research regarding thermoduric bacteria in raw milk and dairy products, the bulk of the

contemporary studies are focused on sporeforming bacteria, resulting in a gap in research concerning non-sporeforming thermoduric bacteria throughout the dairy continuum. To that end, 38 thermoduric bacterial isolates representing nine NSTB genera were used to evaluate variations of the Laboratory Pasteurization Count, the gold standard method for enumerating thermoduric bacteria in milk, including four different heat treatments, two different plating media and three different media incubation times, resulting in 24 possible method combinations. Our goal was to identify a method that would allow for selection of a subset of thermoduric bacteria of practical relevance to the contemporary dairy industry, which will aid monitoring and troubleshooting efforts. Overall, our results indicated that i) there is considerable variability in thermal resistance among NSTB genera, ii) as the temperature of heat treatment increased, the overall cell counts reduced across genera compared to the gold standard heat treatment method, and iii) use of AC Petrifilm underestimates bacterial recovery for some NSTB, compared to gold standard media (i.e., SMA) and should not be used for thermoduric bacteria enumeration.

Thermal resistance within non-sporeforming thermoduric genera varies widely in skim milk broth

Of the nine genera investigated in this study, six contained one or more isolates that exhibited <5 log CFU/mL cell count reduction during the heat treatments applied here (i.e., *Brachybacterium, Microbacterium, Enterococcus, Kocuria, Micrococcus, and Streptococcus*). However, these genera exhibited considerable isolate-to-isolate differences in thermal resistance, with some isolates within the same genus exhibiting >5 log CFU/mL difference in cell count reduction when subjected to the same heat treatment. Interestingly, in the case of the 5 *Microbacterium* isolates tested here, one isolate exhibited a >6 log CFU/mL reduction across all

heat treatments, while the remaining 4 isolates showed minimal destruction (<1 log CFU/mL) across all heat treatments. While studies on NSTB are limited and have primarily focused on identification of thermoduric bacteria in laboratory heat treated raw milk (Ribeiro Júnior et al., 2018; Lee et al., 2024) and not on direct measurement of thermal resistance, there are studies that have identified similar strain-to-strain differences among other dairy relevant bacterial groups, such as pathogens (e.g., *Listeria*, *Salmonella*), the vegetative cells of sporeforming bacteria, and endospores (Ng et al., 1969; Casadei et al., 1998; Janštová and Lukášová, 2001; Besten et al., 2018). For example, Casadei et al., (1998), determined *D*-values for two different strains of *Listeria monocytogenes* (i.e., strain 1151 and Scott A) in four different matrices (i.e., Tryptic Soy Broth, half cream, double cream, and butter) at five different temperatures (i.e., 52°C, 56°C, 60°C, 64°C, and 68°C). The authors determined that the mean *D*-value for strain 1151 over the entire temperature range and for all matrices was nearly double the mean *D*-value for strain Scott A, with mean *D*-values of 870.66 and 467.88 seconds, respectively. Another study, Reich et. al, (2017), evaluated the thermal resistance of vegetative cells of two thermophilic sporeformers (i.e., *Geobacillus stearothermophilus and Anoxybacillus flavithermus).* They enumerated vegetative cell counts before and after heat treatment (i.e., 65°C for 10 minutes, 67° for 5 minutes and 70°C for one minute) for thirteen strains by inoculating strains into UHT skim milk (<0.3% fat), and heating them in a water bath. All strains were isolated from either milk concentrate, pasteurized milk or powdered milk product. Results also identified variability in thermal resistance between strains of the same species. For example, strain 1852 of *A. flavithermus* showed a significant (p<0.05) cell count reduction of 1.5 log CFU/mL after a heat treatment of 67°C for 5 minutes; while the remaining five strains of *A. flavithermus* did not exhibit significant (p>0.05) cell count reductions after the same heat treatment. Similarly,

Geobacillus stearothermophilus strain 35807 showed a significant (p<0.05) cell count reduction of 1.5 log CFU/mL after a heat treatment of 70°C for 1 minute; while the remaining five strains of *Geobacillus stearothermophilus exhibited no significant (p>0.05) reductions after the same treatment. Finally,* Janštová et. al, (2007), evaluated the heat resistance of spores produced by nine different species of *Bacillus* isolated from raw milk and the farm environment reported not only considerable differences in *D*-values between species (i.e., different species of the same genus had different *D*-values) but among species as well (i.e., different strains of the same species had different *D*-values). For example, between spores produced by 21 different strains of *Bacillus licheniformis*, there was a wide range of *D*-values spanning from 2.01 to 11.8 minutes with a mean of 4.51 minutes at 95°C (Janštová et. al, 2007). It is likely that these phenotypic differences in thermal resistance are driven by genotypic features (Besten et al., 2018; Lindsay et al., 2021). Thermal resistance has been studied in other bacterial genera, and has been shown to be related to the presence, or absence of gene(s), or mutations within genes that encode for stress responses (Besten et al., 2018). For example, Mercer et al. (2015) conducted comparative genomics for 29 strains of *Escherichia coli* (*E. coli*) that exhibited phenotypic variability in thermal resistance. In highly heat-resistant strains, a genomic island approximately 14 kb in size, encodes putative heat shock proteins and enzymes, was identified and coined the Locus of Heat Resistance (LHR) (Mercer et al., 2015). This genomic island had previously been identified in thermally resistant strains of *Cronobacter sakazakii* and *Klebsiella pnuemoniae* as well (Bojer et al., 2010; Gajdosova et al., 2011). Similarly, in highly thermal resistant *Bacillus subtilis* strains, a mobile genetic element was identified, that when deleted, conferred heat-sensitive spores (Berendsen et al., 2016). While, to our knowledge, no studies have specifically looked at comparative genomics for non-sporeforming thermoduric bacteria such as those used in this

study, it is likely that the variability observed in this study is driven by similar genetic features, and should be studied further.

Laboratory heat treatment at 70°C for 5 minutes results in a larger cell count reduction in thermoduric bacteria compared to the gold standard method, and selects for a relevant subset of thermoduric bacteria

All genera selected for inclusion in this study had previously been reported to survive some form of heat treatment currently used in the dairy industry, such as the LPC test, vat pasteurization, and in some cases, HTST pasteurization. Literature has documented various genera reported to survive HTST pasteurization at different levels, such as *Microbacterium, Streptococcus, Corynebacterium, Enterococcus, Staphylococcus,* and *Micrococcus* (Washam et al., 1977; Ranieri et al., 2009). The current LPC method for enumerating thermoduric bacteria in milk mimics vat pasteurization, which is utilized far less in the US compared to HTST pasteurization. Therefore, one goal of this study was to identify a variation of the standard LPC method to select for a subset of thermoduric bacteria that exhibit higher thermal resistance, consequently improving the relevance of the test results for HTST pasteurized dairy products.

When comparing heat treatments, our model indicated that heat treatment was nearly significantly (p=0.07) associated with cell count reduction, with heat treatment D (70°C for 5 minutes) exhibiting the greatest reduction in cell count overall. Heat treatment D (i.e., 70°C for 5 minutes) should be further evaluated in naturally contaminated milk samples to fully understand the change in thermoduric populations after this heat treatment, that includes higher temperatures compared to the gold standard method. However, this heat treatment offers a promising

modification of the LPC method to select for a subgroup of higher thermally resistant thermoduric bacteria that are more relevant to HTST pasteurized dairy products.

AC Petrifilm media underestimates bacterial recovery of some genera of NSTB compared to SMA and therefore should not be used for enumerating thermoduric bacteria

Currently, AC Petrifilms are an accepted media for enumeration of thermoduric bacteria (Boor and Martin, 2024). However, our results indicate they can severely underestimate the population concentration of specific non-sporeforming thermoduric genera such as *Kocuria* and *Streptococcus.* These results are similar to other studies that have evaluated AC Petrifilms as an alternative media for enumeration of LPC treated raw milk. For example, Byrne et al., (1991) compared AC Petrifilms to SMA pour plating for laboratory heat treated raw milk. Laboratory heat treated raw milk was inoculated with bacterial cultures of either *Streptococcus, Micrococcus* or *Corynebacterium*. Results showed that out of the seven species of *Micrococcus* tested, six had significantly different ($p<0.05$) counts between media types. Overall, AC Petrifilms underrepresented the bacterial concentration, resulting in differences in counts between the two media ranging from 0.13 log to 2.87 log CFU/mL. The results from Byrne et al., (1991) also illustrate considerable strain-to-strain differences from strains from the same genus. While our results did not indicate significant ($p>0.05$) differences between the two media overall for the genus of *Micrococcus* such as the results of Byrne et al., (1991) did, we do see this pattern of strain-to-strain differences between media for some other genera in our study. For example, at the 48h time point, we observed a >5 log CFU/mL difference in bacterial counts for *Streptococcus* isolates FSL A6-307 and E3-2728 when comparing SMA and AC Petrifilm for the same heat treatment. Specifically, we observed no growth on AC Petrifilm, which would seem to

indicate full thermal inactivation, however, bacterial growth on SMA plates indicated virtually no thermal reduction. For the other three isolates of *Streptococcus* we tested, the two media presented similar results to one another highlighting differences in recovery that can be seen between isolates. These results are also similar to those of Dawkins et. al. (2005), who compared colony counts between dairy products (i.e., powdered skim and whole milk, pasteurized liquid milk, and ice cream) (n=196) plated on AC Petrifilms and SMA media (both incubated at 32°C for 48h) and subsequently identified microorganisms that caused problematic reactions on AC Petrifilms. Their results indicated AC Petrifilms were not a suitable media for dairy products that contained high numbers of Group D *Streptococcus* strains (among other bacteria such as some species of the *Bacillus* genus) due to liquefication of the plate surface or the bacteria's inability to reduce 2,3,5-triphenyltetrazolium chlorine (**TTC**), making enumeration of these colonies nearly impossible. Overall, our results indicate that AC Petrifilms should not be used for enumerating thermoduric bacteria in dairy products due to strain specific reduced recovery and/or enumeration challenges compared with pour plating using SMA

Laboratory thermal resistance studies provide insight into non-sporeforming thermoduric bacteria, however, results may not align with thermal resistance in naturally contaminated dairy products

Accurately representing microbiological communities that reside in complex matrices, as is the case of thermoduric bacteria in dairy products, while minimizing confounding factors is a challenge in microbiology research and could potentially limit generalizability of results (Izurieta and Komitopoulou, 2012). For example, laboratory grown cells have been shown to have different phenotypic characteristics (such as thermal resistance) compared to cells grown

naturally in non-laboratory environments (Washam et al., 1977; Condron et al., 2015). Our own results may point to these challenges. Specifically, previous studies have indicated that *Corynebacterium*, *Macrococcus,* and *Staphylococcus* can withstand the heat treatment utilized in the LPC test (i.e., survive a heat treatment of 63°C for 30 minutes) (Ribeiro Júnior et al., 2018; Lee et al., 2024), yet, in this study, all isolates of *Corynebacterium*, *Macrococcus,* and *Staphylococcus* (*n*=4, *n*=2, *n*=4, respectively) were nearly completely inactivated by all heat treatments including the gold standard LPC heat treatment. Furthermore, all isolates included in this study had originally been isolated from heat treated dairy products, indicating that they had putatively survived that treatment. It is possible that i) these isolates could have been introduced into the dairy product after heat treatment or ii) that NSTBs were in such high concentration pre-pasteurization that while there were surviving cells, it may not indicate a high degree of thermal resistance.

In addition to these factors, we may have observed differences in thermal resistance due to our use of laboratory media (SMB) instead of fluid milk. Skim milk broth is a laboratory media that has commonly been used as a model system for dairy microbiology experiments (Beno et al., 2020), yet there are compositional differences between SMB and dairy products enumerated via the LPC method, namely raw milk. Most importantly, the fat concentration in SMB is 0.5% (Reich et al., 2017), while the average fat concentration in raw milk ranges between 3.4%-5% depending on the breed of cow and other factors (Penn State, 2023). Previous studies have shown that fat level has an impact on thermal inactivation of bacterial cells (Huang et al., 2019; Yang et al., 2021). More specifically, studies have highlighted increased bacterial *D*-values when measured in dairy matrices with higher fat percentages compared to bacterial *D*-values measured within lower fat percentage dairy matrices (Lindsay et al., 2021). For example,

when evaluating the thermal resistance of a *Cronobacter* cocktail (i.e., four *C. sakazakii* isolates and one *C. muytjensii* isolate mixed in equal parts), at temperatures 52°C and 58°C, in different fat percentage dairy matrices (i.e., reconstituted whole milk [3.6% fat], reconstituted low fat milk [1.3% fat] and reconstituted skim milk [0.0%]), Osaili et al. (2009) found that the *D*-value for the *Cronobacter* cocktail was signifcantly ($p<0.05$) larger in reconstituted whole milk indicating increased thermal resistance, compared to the *D*-value within reconstituted low fat or reconstituted skim milk at temperatures 52°C and 58°C under laboratory conditions. For example, the *D*-value of the *Cronobacter* cocktail in reconstituted whole milk at 52°C was 22.10 ± 0.42 minutes while the *D*-value in reconstituted skim milk at 52°C was 15.30 ± 0.24 minutes. On the contrary, however, Read Jr. et al (1961) reported that *E. coli* ATCC 9637 showed a larger *D*-value in raw milk, compared to 40% fat cream (Read Jr. et al., 1961). For example, at a holding temp of 125°C, strain ATCC 9637 over 5 trials had a mean *D*-value of 34.4 minutes within 40% cream compared to a mean *D*-value of 28.2 minutes over five trials at the same holding temp within raw milk. These studies indicate that the discrepancies observed in our results may be influenced by the chosen laboratory matrix used in this study.

Finally, culture preparation methods for laboratory studies on thermal inactivation can affect thermal resistance (Ait-Ouazzou et al., 2012; Condron et al., 2015; Acuff et al., 2021; Lindsay et al., 2021). Parameters of culture preparation methods such as incubation temperature, pH, a_w, and availability of nutrients have been shown to considerably impact the thermal resistance of laboratory grown bacterial cells (Ait-Ouazzou et al., 2012; Condron et al., 2015; Cebrián et al., 2017; Besten et al., 2018). According to Acuff et. al, (2021) when comparing cell preparation methods using Tryptic Soy Broth (**TSB**) compared to Tryptic Soy Agar (**TSA**) lawn preparation, Shiga toxin *E. coli* (**STEC**) strains grown in broth prior to heat treatment had

approximately two times greater *D*-values than STEC strains grown on agar at all temperatures measured in this study (56°C, 59°C and 62°C). For example, strain FNW19M81 (*E. coli* O121:H7), at 56°C had a *D*-value of 17.8 ± 2.7 minutes when cultured in broth, while a *D*-value of 34.5 ± 4 minutes was observed when cells were cultured on an agar lawn. These results are similar to those of Enache et. al (2015), who found that thermal resistance of *Enterococcus faecium* was larger for TSB grown cultures vs TSA grown cultures when inoculated onto talc powder. Specifically, $D_{85°C}$ for TSB-grown cells was 3.42 minutes compared to 2.60 min for TSA-grown cells. In the same study, however, Enache et. al (2015), reports that there was no difference in *D*-values between culture methods for *Salmonella Tennessee*, suggesting that the impact of cell culture preparation may also be strain dependent. In our study, the final media in which cultures were prepared prior to inoculation and heat treatment was a lawn on an agar plate which has a lower a_w than broth, which could have affected the final thermal resistance of the isolates used here, regardless of the isolates being previously recovered after some degree of heat treatment. Overall, while these limitations of laboratory thermal resistance studies have likely impacted the generalizability of our results, we believe our study provides important insight into the phenotypes of commonly found NSTB in the dairy system.

CONCLUSION

The results from this study expand on the limited knowledge of non-sporeforming thermoduric bacteria relevant to the dairy supply chain, while highlighting the wide variability in thermal resistance between strains at temperatures similar to those encountered in dairy manufacturing. Here, we offer a modified laboratory heat treatment method that selects for a subgroup of NSTB that are more likely to survive HTST pasteurization, therefore improving the relevance of the outcomes of this modified test to dairy industry community members. Future

studies should examine this method in naturally contaminated raw milk and other dairy products. Our results also demonstrate that strain-to-strain variability in thermal resistance within thermoduric genera is not uncommon, thus highlighting the need for more research to understand the genotypic features driving this variability. Overall, our results are also of practical importance to the dairy industry as they indicate that there is reduced recovery of some thermoduric bacteria on media used to enumerate these organisms (i.e., Aerobic Count Petrifilm), and 48h of incubation is appropriate for recovery of NSTB following laboratory heat treatment.

NOTES

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SUPPLEMENTAL MATERIAL

Supplemental Figure 3.1. Rain drop plot showing reduction in cell count (log CFU/mL) for every strain at every combination of method parameters (4 heat treatments, A-D [A: 63°C for 30 minutes, B: 65°C for 15 minutes, C: 68°C for 7 minutes, D: 70°C for 5 minutes], 2 media types (Standard method Agar [SMA]) and 3M Aerobic Count Petrifilms [PF]) and 3 incubation times.

CHAPTER IV

ASSESSMENT OF DRIVERS OF *LISTERIA* ENVIRONMENTAL MONITORING PROGRAMS IN SMALL AND MEDIUM-SIZED DAIRY PROCESSING PLANTS*

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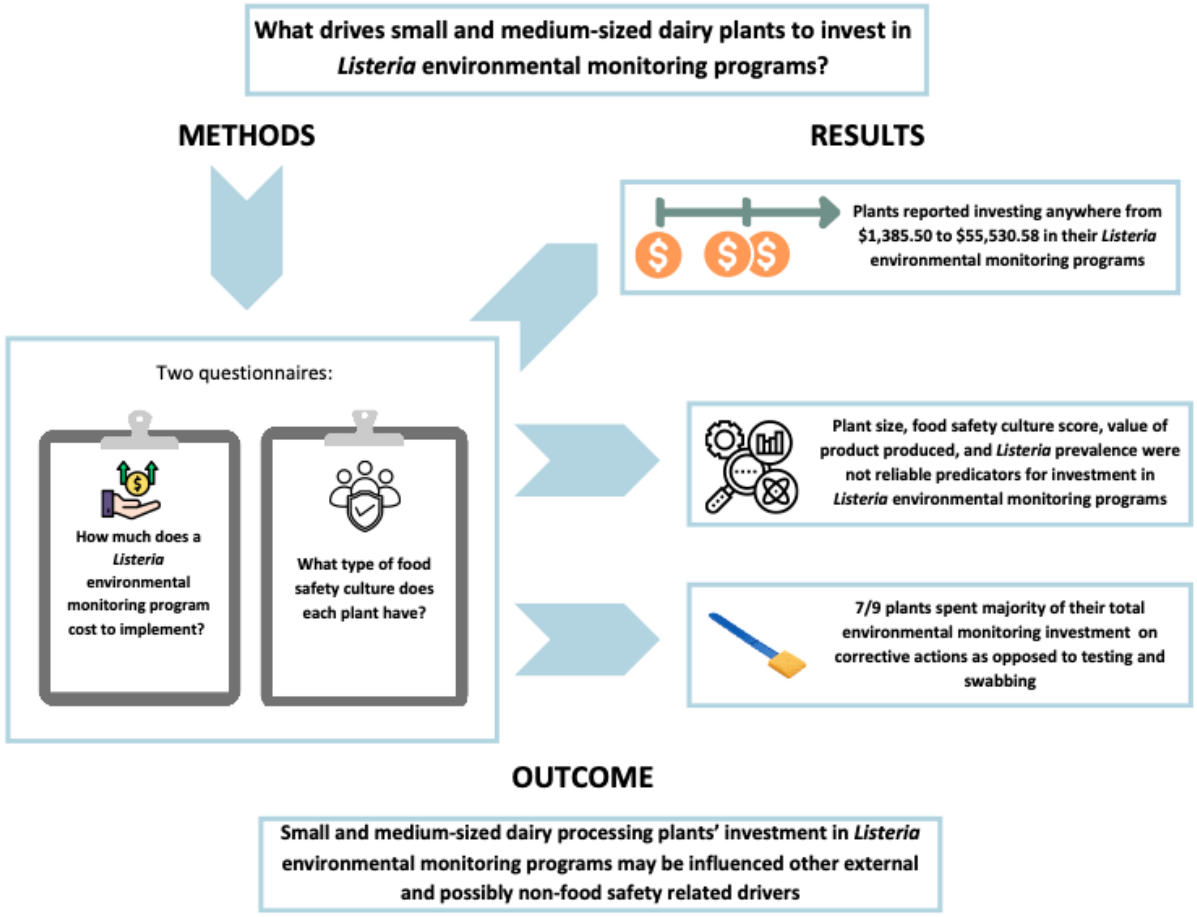
* P*repared for submission to Journal of Dairy Science Communications*

GRAPHICAL ABSTRACT

Figure 4.1 Graphical abstract highlighting key findings from investigation into what drives small and medium-sized dairy plants to invest in *Listeria* environmental monitoring programs



SUMMARY

Small and medium-sized dairy processing plants (SMDPPs) may face unique challenges in implementing *Listeria* Environmental Monitoring Programs (EMPs). To understand their EMP investment decisions, we evaluated 9 SMDPPs for both i) their food safety culture and commitment to *Listeria* EMP implementation, and ii) financial costs associated with their *Listeria* EMP (e.g., costs of swabbing and testing, costs of corrective actions, and estimated values of the total finished product). Corrective actions accounted for majority of total EMP

investment in 7 plants, highlighting possible prioritization of corrective actions by SMDPPs. A linear model identified that plant size, overall *Listeria* prevalence, estimated total value of finished product and food safety culture were not reliable predictors of total EMP investment, suggesting the influence of external, potentially non-food safety related factors. Further evaluations of EMP investment drivers and food safety economics are needed to develop targeted strategies for food safety support.

HIGHLIGHTS

- 9 dairy plants were evaluated for costs associated with their *Listeria* EMPs
- Total *Listeria* EMP investment varied widely across plants
- Corrective actions accounted for majority of total EMP investment for 7/9 plants

ABSTRACT

Small and medium-sized dairy processing plants (**SMDPP**s) may face unique challenges, such as limited financial resources, when implementing *Listeria* environmental monitoring programs (**EMP**s). To better understand what drives investment into *Listeria* EMPs, 9 SMDPPs, which previously participated in a ~1-year longitudinal study focused on implementing *Listeria* EMPs, completed a questionnaire regarding their EMP-associated costs. This questionnaire was distributed to each SMDPP via e-mail and was used to obtain each plant's (i) self-reported product value (i.e., estimated total value of finished product in a plant at any given time [**ETVFP**]) and other EMP associated costs (e.g., corrective action costs), as well as (ii) perceived ability to control pathogens in their processing environment. Total EMP investment refers to the total amount of money that plants spent on their *Listeria* EMP, and was calculated as the sum of calculated swabbing and testing costs and self-reported corrective action costs. Additionally, at

the end of the study period, we utilized a pre-defined rubric to assess each SMDPP's commitment to their *Listeria* EMP; these data were used to obtain a "food safety culture score" for each SMDPP out of 225 possible points. Overall, a wide range of total EMP investments ($1,388.50 to $55,530.58) and food safety culture scores (56/225 to 222/225) were reported across plants. Additionally, for 7/9 plants, corrective action costs accounted for >50% of their total EMP investment, indicating the possible prioritization of corrective actions by SMDPPs. There was no association between percentage of total EMP investment that a given plant allocated toward corrective action costs and the change in *Listeria* prevalence in the follow-up sampling event compared to the initial sampling event. Overall, linear modeling identified that predictor variables of plant size, overall environmental *Listeria* prevalence during the EMP implementation period, ETVFP, and food safety culture score were not significantly associated with the outcome of "total EMP investment," suggesting that SMDPPs' financial investments into *Listeria* EMPs may be influenced by other external or non-food safety related factors. Therefore, further research is needed to more thoroughly examine a wider range of factors that could possibly drive EMP investment and food safety culture in SMDPPs. Such efforts will be highly valuable towards developing more targeted strategies to enhance the implementation of *Listeria* EMPs and other environmental pathogen control strategies in SMDPPs and other small and medium-sized food business operations.

MANUSCRIPT

Listeria monocytogenes (**Lm**) is a foodborne pathogen that causes foodborne listeriosis, a bacterial infection that is associated with high mortality and hospitalization rates, making it a considerable public health concern (European Food Safety Authority, 2018). Importantly, Lm can persist in the environments of food processing plants, including equipment, floors, and drains

(Leong et al., 2014; Bolten et al., 2024a), increasing the potential for contamination of ready-to-eat (**RTE**) foods for human consumption with Lm (Cartwright et al., 2013). To address this public health concern, regulation, third parties, and various internationally recognized food safety frameworks (e.g., Brand Reputation through Compliance [BRC] Global Standard) highlight *Listeria* environmental monitoring programs (**EMP**s) as a tool to better control Lm and other *Listeria* spp. in food processing environments based on risk (Zoellner et al., 2018; Brand Reputation through Compliance Global Standard, 2022). Notably, several recent listeriosis outbreaks and Lm recalls have implicated RTE dairy products (e.g., cheese, ice cream) manufactured by small and medium-sized dairy processing plants (**SMDPP**s) (Centers for Disease Control and Prevention, 2008, 2022; U.S. Food and Drug Administration, 2017a, 2023b). Importantly, all plants implicated in these outbreaks and recalls either i) did not have *Listeria* EMPs in place (Centers for Disease Control, 2008; U.S. Food and Drug Administration, 2022) or ii) had *Listeria* EMPs that were deemed ineffective at controlling Lm persistence in their processing environments based on regulatory inspectional observations (U.S. Food and Drug Administration, 2017b; 2023a).This highlights the importance of continuing research and improvement efforts geared towards implementation of proper *Listeria* EMPs in SMDPPs.

However, SMDPPs may face unique challenges related to implementing *Listeria* EMPs. For example, *Listeria* EMPs in SMDPPs may receive insufficient monetary investment due to i) limited financial resources (Bhaskaran, 2006; Nayak and Waterson, 2017; Lee et al., 2023; Magiya, 2023), or ii) possible underappreciation of food safety programs due to poor food safety culture, which is defined as a set of shared values or beliefs that drive actions surrounding food safety practices within a given company (Yiannas, 2009; Griffith et al., 2010). Food safety culture goes beyond the immediate food safety programs within a company (e.g., policies,

training, audits) and incorporates communication and management support (Powell et al., 2011). Notably, a lack of food safety culture is frequently reported as an environmental antecedent, or underlying root cause, of various foodborne illness outbreaks (Wittenberger and Dohlman, 2010; Powell et al., 2011; Holst et al., 2024). For instance, in a 2008 *Salmonella* outbreak linked to products produced by Peanut Corporation of America, poor food safety culture among company officials resulted in several negligent food safety practices, such as shipping products that tested positive for *Salmonella Typhimurium* and falsifying Certificates of Analyses (**COA**s) documents to conceal positive results from customers (Medus et al., 2009).

An important aspect of making food safety programs practical and enticing for businesses is to first understand what drives a business to invest financial resources into food safety programs. Currently, there is limited research characterizing the financial investments that food businesses put towards their food safety programs, such as *Listeria* EMPs, possibly due to the sensitive nature of financial data (Gomez & Marks, 2020). Moreover, while several studies have sought to assess the food safety culture of a variety of food business operations (Zanin et al., 2021), few have assessed food safety culture in smaller establishments (Nayak and Waterson, 2017), and none (to the authors knowledge) have further evaluated the relationship between food safety culture and financial investment into *Listeria* control programs for smaller establishments. Thus, the goals of this study were to i) provide a benchmark for how much SMDPPs are investing into *Listeria* EMPs, and ii) identify what drives SMDPPs to invest in *Listeria* EMPs, so that ultimately, food safety interventions can be better targeted at SMDPPs. Towards this end, we evaluated 9 SMDPPs, which had previously implemented *Listeria* EMPs in a ~1-year longitudinal study (Bolten et al., 2024a; 2024b), using two different questionnaires to assess both

i) their food safety culture and commitment to *Listeria* EMP implementation as well as ii) their financial investment into *Listeria* EMPs.

The 9 SMDPPs evaluated in this study are located in the Northeastern United States, and primarily manufacture either fluid milk (plants CL, N and W), cheese (plants CM, BY, CQ) or ice cream (plants CN, CO, CP). Plants N and W were classified as "medium-sized" processing plants (i.e., processing between 1 million and 100 million lb. of milk solids from raw milk per year) and the remaining 7 were classified as "small-sized" (i.e., processing < 1 million lbs. of milk solids from raw milk per year). All 9 SMDPPs previously participated in a ~1-year longitudinal study focused on implementing and evaluating *Listeria* EMPs for *Listeria* control, which involved collection and testing of environmental sponges for *Listeria* detection by the research team through both i) "initial sampling events" prior to *Listeria* EMP implementation and ii) "follow-up sampling events" ~1 year after implementation as described by Bolten et al. (2024a) for 8 plants (i.e., plants CL, CM, CN, CO, CP, CQ, N, and W) and by Bolten et al. (2024b) for plant BY.

We collected information on the food safety culture and *Listeria* EMP-related costs of the 9 SMDPPs using two distinct questionnaires. The first questionnaire, which assessed SMDPPs' food safety culture and overall dedication to *Listeria* EMP implementation, was administered to 5 members of the research team who worked closely with the SMDPPs during the *Listeria* EMP implementation period, via a Qualtrics Survey (Qualtrics, Provo, UT) ~1 month after the longitudinal *Listeria* EMP study had concluded. This questionnaire was composed of 9 descriptive statements pertaining to how proactive a given plant was at carrying out *Listeria* EMP activities (e.g., swabbing, corrective actions) and the attitude of management and other employees towards food safety. For each plant, research team members were asked to rate their

level of agreement with each of the 9 descriptive statements on a 5-point Likert scale (Likert, 1932) (i.e., 1: strongly disagree, 2: disagree, 3: neither agree nor disagree, 4: agree, 5: strongly agree). All research team member ratings across the 9 statements were summed together to yield a "food safety culture score" of up to 225 possible points for each plant. The second questionnaire, which assessed SMDPPs' *Listeria* EMP financial investments and overall perception of pathogen control, was administered to SMDPPs via email ~1 year after the longitudinal *Listeria* EMP study had concluded. This questionnaire was composed of both short answer and multiple choice questions, where SMDPPs were instructed to i) report several financial metrics (in U.S. dollars), including the estimated cost of corrective actions during the 1-year *Listeria* EMP implementation period and the estimated total value of finished product (**ETVFP**) that typically is in storage in the plant on any given day (we assumed that this would be an appropriate proxy measure for the financial risk associated with a possible recall), as well as ii) rate their perceived control over pathogens in their plant after participating in the longitudinal *Listeria* EMP study on a scale of 1-5 (i.e., 1: No control, 2: Little control, 3: Some control, 4: Mostly under control, 5: Full control). Participation in this questionnaire was completely voluntary and no compensation was provided. Both questionnaires, along with raw data, can be accessed in a publicly available repository (https://github.com/FSL-MQIP/Cost-of-EMP.git).

All data obtained from questionnaires was formatted and cleaned to facilitate statistical analysis. For example, if a plant reported a range of values for a given short answer question, the mean of the two values was used for analyses (e.g., if "$80,000-100,000" was reported, $90,000 was used for analysis). In cases where a plant reported spending less than a given value for a given short answer question, a value of 1 unit lower than the reported value was used for

analyses (e.g., if "<$5,000" was reported, $4,999 was used for analysis). Estimated environmental swabbing and testing costs for each SMDPP were calculated by multiplying the number of environmental sponge samples that were collected and tested by individual plants during routine sampling events in the longitudinal *Listeria* EMP study (Bolten et al., 2024a; 2024b) by the estimated cost of sampling materials (i.e., environmental sponge swabs, $1.50-$1.86 depending on sponge type) + laboratory testing fees ($20.00 per swab). The value of "Total EMP investment," defined as the costs associated with a given plant's implementation of *Listeria* EMP over the ~1 year period, was then calculated for each plant by adding estimated swabbing and testing costs + estimated cost of corrective actions (reported in the *Listeria* EMP-related costs questionnaire). In addition to questionnaire data obtained from the current study, we also utilized previously reported *Listeria* prevalence data from the 1-year longitudinal *Listeria* EMP study (Bolten et al., 2024a; 2024b) in statistical analyses to assess i) if overall *Listeria* prevalence (the percent of environmental sponge samples that tested positive for *Listeria* across initial and follow-up sampling events) was associated with the outcome of "total *Listeria* EMP investment", ii) if changes in *Listeria* prevalence after 1-year of EMP implementation (reported here as the percentage of *Listeria* positive samples in the follow-up sampling event – the percentage of *Listeria* positive samples in the initial sampling event in each plant) was influenced by the predictor of "investment into corrective actions", and iii) if *Listeria* prevalence in the follow-up sampling event was correlated with plants' self-reported perceived control over pathogen presence at the end of the study period (Table 4.1).

Statistical analysis was performed in R version 4.3.3. A linear regression model was used to test the effect of ETVFP, annual lbs. of milk solids processed from raw milk, overall *Listeria* prevalence, and food safety culture score on the outcome of "total EMP investment" (i.e., the

total dollar amount investment by a given plant into their *Listeria* EMP); total EMP investment values were log transformed to satisfy normality assumptions. Additionally, a linear regression model was used to assess the effect of investments into corrective actions (i.e., the percentage of total EMP investment that was allocated specifically to corrective actions) on the outcome of "change in *Listeria* prevalence in the follow-up sampling event compared to the initial sampling event". Finally, Pearson's product moment correlation test was conducted to compare plants' perceptions of their own pathogen control to follow-up *Listeria* prevalence. Alpha was set to 0.05 for all analyses to denote significant difference, effect or association.

Results from questionnaires showed wide ranges in both ETVFP and EMP-related costs. ETVFP values ranged from $7,500 to $2,200,000 (i.e., plants CO and N, respectively), with 7/9 plants reporting ETVFPs of ≤$100,000 (i.e., plants BY, CL, CO, CQ, CM, CN and CP) (Table 4.1).

Table 4.1. Environmental monitoring program (EMP) associated costs, *Listeria* prevalences (Bolten et. al, 2024a; 2024b), food safety culture scores, and pathogen control perceptions reported for 9 small and medium-sized dairy processing plants (SMDPPs)

		EMP associated costs[b]			*Listeria* prevalence[c]				
Plant	ETVFP in $[a]	Total EMP investment in $	Corrective action cost in $ (% of total EMP investment)	Swab and testing cost in $	Overall (%)	Follow-up (%)	Δ (% points)	Food safety culture score (out of 225)[d]	Plant's perceived control over pathogens after *Listeria* EMP implementation (out of 5)[e]
BY	10,000	25,903	25,000 (97)	903	29	8	-42	190	4
CL	20,000	2,215	2,000 (90)	215	28	25	-5	79	3
CM	100,000	5,796	<5,000 (86)	796	3	3	0	198	2
CN	100,000	5,215	5,000 (96)	215	39	15	-48	100	3
CO	7,500	1,389	980 (71)	409	15	20	10	122	4
CP	200,000	5,000	5,000 (100)	0	19	36	34	56	3
CQ	40,000	1,187	400 (34)	787	12	15	7	166	4
W	**80,000-100,000**	55,531	50,000 (90)	5,531	27	22	-9	129	4
N	2,200,000	12,485	200 (2)	12,285	3	6	5	222	4

[a] ETVFP estimated total value of all finished product in a plant at any given time, which was self-reported by plants in the EMP cost questionnaire.
[b] Total EMP investment represents the costs associated with a given plant's implementation of *Listeria* EMP over the ~1 year period; it was calculated as the sum of swab and testing costs and self-reported corrective action costs. Swab and testing costs were calculated by multiplying the number of environmental sponge swabs used by a given plant as part of their routine EMP sampling (Bolten et al., 2024a; Bolten et. al., 2024b) by the estimated costs of sampling materials (i.e., environmental sponge swabs, $1.50-$1.86 depending on sponge type) and laboratory testing fees ($20.00 per swab).
[c] Overall *Listeria* prevalence refers to the percent of environmental sponge samples that tested positive for *Listeria* across initial and follow-up sampling events. Follow-up *Listeria* prevalence refers to the percent of environmental sponge samples that tested positive only at the follow-up sampling events. (Δ) represents the change in *Listeria* percent of positive environmental sponge samples in follow-up sampling event compared to the initial sampling event. All *Listeria* prevalence data were obtained in two previous studies; for plants CL, CO, CQ, CM, CN, CP, N and W, see (Bolten et al., 2024a) for details; for plant BY, see (Bolten et al., 2024b) for details.
[d] Indicates the research team's perception of the plant's dedication to EMP implementation and overall food safety culture. Five research team members ranked each plant on 9 statements associated with food safety culture using the Likert scale (scale of 1-5). Higher scores indicate stronger EMP dedication and food safety culture.
[e] Self-reported by plants. Each plant chose one of the following statements which is associated with a point value: 1. No control, 2. Little control, 3. Some control, 4. Mostly under control and 5. Full control

Total EMP investment values ranged from $1,187 to $55,531 (i.e., plants CQ and W, respectively), with the majority (6/9) of plants investing <$10,000 into their *Listeria* EMP (i.e., plants CL, CM, CN, CO, CP, and CQ). Finally, food safety culture scores ranged from 56/225 to 222/225 (i.e., plants CP and N, respectively), with the majority (6/9) of plants receiving food safety culture scores ranging between 100 and 198/225 (i.e., plants BY, CM, CN, CO, CQ and W).

Linear regression analysis showed that there was no significant association between the predictor variables of ETVFP, plant size (i.e., annual lbs. of milk solids processed from raw milk), overall *Listeria* prevalence, and food safety culture score on the outcome of "total EMP investment" (p=0.39), indicating that none of these factors could represent reliable predictors for how much money SMDPPs are willing to invest into their *Listeria* EMPs. This could indicate that other variables, potentially ones that are less obviously linked to food safety, may be driving SMDPPs' financial decision making regarding their food safety programs. While few studies focused on identifying drivers of food safety investments in smaller establishments have been conducted, indicating a gap in research, one study (Yapp and Fairman, 2004), which conducted interviews with 50 small- and micro-food businesses, identified enforcement by regulators as a primary driver of food safety investments, which was not measured in our study. While pressure to comply with regulatory requirements represents a potential driver that is likely linked to food safety, it is important to note that decision making in small and medium sized food businesses can oftentimes be solely driven by the beliefs and attitudes of a single owner (Bhaskaran, 2006), who may be more likely to rely on "gut feelings" or other non-food safety related factors to drive decision making (Vos and Vos, 2000; Danielson and Scott, 2006). For example, Danielson and Scott (2006) compiled survey data by the National Federation of Independent Business to

analyze the strategies small businesses use to evaluate business financial expenditures, and found that small businesses tend to use less sophisticated expenditure evaluation tools, more often relying heavily on owners' gut feelings to evaluate expenditures. Furthermore, the authors hypothesize that possible reasons for differences between large and small businesses in business expenditure analyses methods could be due to i) limited educational background in small businesses, ii) limited staff size, iii) liquidity concerns, and iv) cash flow estimation challenges. Therefore, it is possible that similar variables such as gut feelings from owners and upper management could also represent drivers of *Listeria* EMP investment for some of the SMDPPs examined in this study. This highlights a larger issue of small business owners potentially using gut feeling to drive other food safety related decisions such as determination of food safety strategies or corrective actions instead of using data driven decision making. This, in turn, could lead to negative public health related consequences (e.g. outbreaks). Therefore, trainings targeted towards owners and upper managements of small food businesses surrounding the value of data driven food safety strategies are imperative.

SMDPPs reported allocating varying percentages of their total EMP investment towards swabbing and testing costs vs. corrective action costs. For example, 7/9 plants (i.e., plants BY, CL, CM, CN, CO, CP and W) spent more than 50% of their total EMP investment on corrective actions, with plant CP reporting to spend the highest percentage (100%), followed by plant BY (97%), and plant CN (96%) (Table 4.1). Plant N reported spending the lowest percentage of their total EMP investment on corrective actions (2%) followed by plant CQ (34%) and plant CO (71%). Notably, these three plants had increases in *Listeria* prevalence in the follow-up sampling event compared to the initial sampling event (increases of 5, 7 and 10 percentage points in *Listeria* prevalence for plants N, CQ, and CO, respectively), which might, anecdotally, suggest,

that limited investments into corrective actions can have negative food safety outcomes (i.e., increased *Listeria* prevalence). However, linear regression analysis showed that, across all plants, there was no statistically significant association (p=0.32) between the percentage of total EMP investment that a given plant allocated toward corrective action costs and the change in *Listeria* prevalence in the follow-up sampling event compared to the initial sampling event.

Since investment into corrective actions was not associated with a decrease in *Listeria* prevalence, this could highlight that the effectiveness of a corrective action does not lie solely with its financial cost. Similarly, other studies in which corrective actions had limited effect on improvement of microbial food safety or quality related issue exist as well (Etter et al., 2017; Reichler et al., 2020). For example, Reichler et al. (2020) conducted a case study regarding the efficacy of 3 intervention strategies targeted towards reducing post-pasteurization contamination (**PPC**) in high-temperature, short-time-pasteurized **(HTST)** fluid milk in 4 large dairy plants located in the Northeast. The three strategies were i) employee training, ii) employee training combined with modified clean-in-place chemistry, and iii) preventive maintenance focused on replacement of wearable rubber parts. They found that strategies i and ii did not significantly decrease PPC while strategy iii may have reduced the frequency of PPC (reduction of PPC in 3/3 samples before implementation to 1/3 samples after). While the costs of these strategies were not evaluated in this study, the authors postulated that possible reasons for the ineffectiveness of strategies i and ii on reducing PPC were lack of buy-in from management, or misidentification of the root cause. It is possible, that in our study, SMDPPs' faced similar problems regarding their own corrective actions that could have affected effectiveness. Additionally, while our study did not capture preventive actions, they are an integral part of an EMP (Neogen, 2023) and while corrective actions can potentially reduce environmental pathogen prevalence (Barnett-Neefs et

al., 2022), it may be useful for industry to take it a step further and shift towards an even more preventive approach that utilizes both corrective actions and preventive actions. Future studies estimating effectiveness of corrective actions should consider incorporating aspects such as buy-in from management or preventive actions into their scope while continuing to assess costs as well.

Another consideration in our study is the possibility that SMDPPs misunderstood the meaning of technical EMP terms such as "corrective action" and thus reported inaccurate responses. For example, in our study, plant CP reported spending $5,000 on *Listeria* EMP related corrective actions and yet, according to Bolten et al. (2024a) there was no record of them conducting any environmental swabbing during the year. This could point to a larger issue, such as small and medium plants' (often theorized to have less food safety experience than larger plants [(Lee et al., 2023)]) possible misunderstanding of more specialized food safety terms such as "corrective action," especially when other seemingly similar terms like "preventive action" exist in the industry. In fact, (Charalambous et al., 2015) reports that maximum hygiene improvement in small food businesses in Cyprus coincided with simpler food safety management systems compared to more complex systems, highlighting the importance of simple, easy to understand, food safety management systems for small businesses. Although some studies have focused on food safety training gaps specifically for small food businesses (Holt & Henson, 2000; Walker et al., 2003) these studies tend to focus on more basic topics, such as food hygiene, and good manufacturing practices instead of more technical areas like practical guidance on specialized EMP-specific concepts and procedures, thus highlighting a gap in research and resources for small food businesses. Additionally, such practical guidance on specialized EMP-specific concepts could include a focus on effective corrective actions, taking into account

previously mentioned factors such as buy-in from upper management, costs, and proper identification of root causes.

Interestingly, some responses from the EMP-related costs questionnaire revealed that some SMDPPs may have inaccurate perceptions of their own pathogen control. For example, the plant that received the lowest food safety culture score and showed the highest *Listeria* prevalence in the follow-up sampling event (i.e., plant CP) perceived their control over pathogens after ~1 year of *Listeria* EMP implementation as "Mostly under control" (i.e., 4/5 on a 5-point scale), which was the same response reported by the plant that received the highest food safety culture score and the lowest *Listeria* prevalence in the follow-up sampling event (i.e., plant N) (Table 4.1). Moreover, a Pearson's coefficient correlation test comparing plants' perception of their own pathogen control to *Listeria* prevalence observed in each plant's follow-up sampling event identified a low correlation coefficient of -0.31, potentially indicating plants' having inaccurate perception of their own pathogen control, however, this result was not statistically significant (p= 0.41). Given that the 95% confidence interval for this test was so large (i.e., -0.80 to 0.45), the lack of statistical significance could be due to the small sample size, which highlights a key limitation that should be considered when interpreting this and other statistical analysis results from this study. Furthermore, other factors, such as potential over reporting of socially desirable values (i.e., investing more into corrective actions, perceiving pathogen control as better than it is) by SMDPPs should also be considered. In other words, plants may want to appear that they are "doing the right thing" without having to do the right thing, especially if they have poor food safety culture (e.g., plant CP, which received the lowest food safety culture score [56/225] in this study). Studies identifying reporting bias in favor of social norms (in both food safety and other contexts) are not uncommon (Jenner et al., 2006;

Ungku Fatimah et al., 2014; Banna et al., 2015; da Cunha et al., 2019). For instance, da Cunha et al. (2019) created two structural equation models based on data collected from 183 food handlers in Brazil, including one which represented data on food safety practices self-reported by food establishments, and another which represented data on those same food safety practices that were observed by outside food safety inspectors. Findings showed a weak correlation (r= 0.33, p=0.002) between the two models, indicating a discrepancy between self-reported and observed food safety behaviors, with the self-reported behaviors seeming to be overinflated compared to the observed behaviors. Furthermore, one study (Jespersen et al., 2017) developed and validated a formal scale to capture social desirability in food safety culture. Future studies incorporating self-reported food safety behaviors should take into account social desirability so that results are accurate and interventions aimed at improving food safety culture can be better targeted.

Overall, as specific drivers of SMDPPs investment into *Listeria* EMPs could not be identified in this study, further research aimed towards understanding what drives small and medium businesses to invest in food safety programs using a larger sample size is advised. Additionally, SMDPPs seem to prioritize corrective actions as exemplified by their spending habits, however effectiveness of corrective actions may be associated with other variables such as buy-in from management or proper identification of root causes. Additionally, limitations such as possible misinterpretation of food safety related terms like corrective actions, and bias from self-reporting may have influenced results. Finally, this study can serve as a starting point for further research regarding economic investment into food safety or food safety culture, and in doing so, results may help better tailor food safety interventions to small and medium sized food processing plants.

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CHAPTER V

CONCLUSIONS

As academia continues to conduct integral research developing food safety and quality related tools for industry, it is imperative that such tools are able to be effectively implemented into the business context. To do this, we conducted three investigations to i) identify challenges with implementation of food safety and quality related tools into industry (in the context of applying AI for food safety in the CEA industry), ii) create a practical and easily applicable quality-related tool for industry (in the context of developing microbiological testing for defect causing bacteria in the dairy industry) and, iii) work to close research gaps regarding the effective implementation of food safety tools such as costs of food safety programs and recalls (in the context of small and medium dairy plants).

For chapter 2, we collaborated with experts in CEA and AI from Wageningen University in the Netherlands and Centro de Edafología y Biología Aplicada del Segura (CEBAS) in Spain to organize a workshop focused on the challenges and opportunities of using AI to manage microbiological food safety in CEA operations. Additionally, we visited a CEA facility in Spain to gain deeper insights into microbiological food safety hazards specific to CEA. We identified that there are currently still many challenges that stand in between the CEA industry and its effective implementation of AI as a food safety tool. Challenges involve i) a need for research investigating the contamination sources, routes and microbial environment of known and unknown pathogens in a variety of CEA facilities, ii) a need for stronger basic sanitation and hygiene practices in CEA, iii) the need for generation of a vast amount of data, iv) the wide variety in CEA facilities and data infrastructures and, v) the need to communicate the financial returns from using said tools to decrease recall risks and improve overall food safety. From this,

we can learn that effective tools for industry need to take into account the industry's current infrastructures and food safety/quality maturity level and the need to translate improved food safety (i.e., reduction in risk of recalls) to financial returns.

In Chapter 3, we worked to apply some of these findings by creating a microbiological testing method to enumerate non sporeforming thermoduric bacteria along with furthering research regarding their thermal resistance. To do so, 38 isolates from non-sporeforming genera were inoculated into skim milk broth and independently subjected to four different heat treatments (A: 63°C for 30 minutes, B: 65°C for 15 minutes, C: 68°C for 7 minutes and D: 70°C for 5 minutes), followed by plating using two different media types (Standard Methods Agar and Aerobic Count Petrifilms), each of which were incubated and enumerated after three different incubation periods (24h, 48h, and 72h) at 32°C. Beside the wide variety of thermal resistances among these isolates, an interesting outcome was that Aerobic Count Petrifilms are not a suitable media to recover some non sporeforming thermoduric bacteria on. Additionally, while a 72h incubation allowed for more recovery of bacterial colonies, the 48h incubation was chosen because we believed the time savings that businesses would experience was more valuable than the minimal increase in recovery.

Finally, in Chapter 4, we aimed to conduct initial research on some of the gaps regarding financial costs of food safety tools and costs of recalls that we outlined as a challenge for implementation in Chapter 2, within the context of small and medium dairy plants and their *Listeria* EMPs. To do this we questioned 9 small and medium dairy plants regarding their costs of EMP implementation and their perceived control over pathogens. Additionally, the research team assigned a food safety culture score to each plant. We identified that small and medium businesses may use non-food safety related drivers for EMP investment such as "gut feeling,"

and investment into EMP varied considerably. There were limitations with this study, however, such as possible bias in self-reporting and small sample size. Future studies should aim to conduct similar research using larger sample sizes and can use this chapter as a starting point.

Overall, in order for food safety and quality related tools to be most effective for industry, they need to utilize current industry infrastructure and be able to convey financial returns to businesses. To that end, the work provided in this thesis provides insight into i) how to create effective and practical food safety and quality tools for industry and, ii) conducting research to quantify costs and savings for said tools.