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Effect of pre-growth conditions on foodborne pathogen survival kinetics in fresh produce

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EFFECT OF PRE-GROWTH ENVIRONMENTAL CONDITIONS ON FOODBORNE PATHOGEN SURVIVAL KINETICS IN FRESH-CUT PRODUCE

A Thesis

by

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ABSTRACT

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Worldwide consumption of fresh-cut produce has seen a dramatic increase over the past few decades. However, fresh-cut produce has been implicated in several illness outbreaks due to contamination with foodborne bacterial pathogens. Post-harvest contamination during handling and processing is considered rare but several influencing factors are often overlooked. Specifically, studies have reported that the growth, survival, and death kinetics of foodborne bacterial pathogens such as *E. coli* O157: H7, *Salmonella,* and *Listeria monocytogenes* in different fresh and fresh-cut produce. The effect of various factors such as storage temperature, time, and relative humidity are widely explored. It is increasingly evident that microbial pathogens are naturally exposed to various environmental stresses in the farm-to-fork continuum. The nature of stresses and their response to these stresses determine the likelihood of risk. Limited knowledge exists in understanding the effect of environmental stresses on the growth and survival kinetics of foodborne pathogens. Thus, this thesis research mainly focused on understanding the effect of select environmental stresses on the survival kinetics of *Salmonella enterica* and *L. monocytogenes*. A study was conducted to determine the effect of different pre-

growth temperatures (4, 21, and 37 ºC) on survival kinetics of *S. enterica* and *L. monocytogenes* in fresh-cut salad during simulated retail or consumer refrigerated storage at 4 ºC and 80 % RH. Pre-growth temperature(s) and the type of produce showed a significant ($p \leq 0.05$) effect on the survival kinetics of *L. monocytogenes* during storage. Among the tested produce, mixed salad and shredded carrot showed the highest reduction of *L. monocytogenes* and *S. enterica*, respectively. Baranyi and Roberts model better fitted the experimental data (R^2; 0.054 to 0.967 for *L. monocytogenes*, and 0.194 to 0.994 for *S. enterica*). Another study was conducted to determine the effect of pre-growth environmental conditions (4 and 37 ºC, pH 4.5) on the responses of *L. monocytogenes* to different antimicrobial light (UVC, UVA, and blue light) treatments. As expected, increasing the treatment time increased the log reductions. Under the tested conditions pre-growth stresses did not show a significant ($P>0.05$) effect on the *L. monocytogenes*. These findings indicate that appropriate selection of pre-growth environmental conditions is critical to better understand the survival kinetics of foodborne pathogens in fresh-cut produce and to develop efficient interventions to mitigate the risk.

Keywords: *L. monocytogenes*; *Salmonella;* Fresh-cut produce; Environmental conditions; Modelling; Light treatment

DEDICATION

I dedicate this thesis to my mother Mrs. Jaspal Kaur, my father, Mr. Narinder Pal Singh, sister Navrinder Kaur and Brother-in-law Jagmeet Singh, who always taught me to work hard and keep going. I hope that this achievement will complete the dream that you had for me all those years ago when you chose to give me the best education you could. To my beloved brother-in-law Jagmeet Singh, in loving memory. I hope you would be very proud of me.

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CHAPTER I

INTRODUCTION

The popularity of fresh-cut fruits and vegetables has seen a meteoric rise in the recent past owing to their fresh, convenient, minimally processed, and health-promoting properties (Miranda et al., 2016; Miceli & Settanni, 2019; Almenar, 2020). They are rich sources of vitamins, minerals, dietary fiber, and phytochemicals that are known to mitigate the risk of chronic diseases such as diabetes, cardiovascular disease, hypertension, and cancer (Zhang et al., 2015; Tango et al., 2018; Aune, 2019). When it comes to fresh-cut produce quality and safety are the two primary determinants of consumer acceptability and the safety of public health. Between 2017-2020, a total of 1422 outbreaks were attributed to fresh produce that includes both intact (whole) and non-intact (fresh cut) categories (CDC, 2022). Due to minimal or no-processing treatments involved compounded with accelerated enzymatic activity and susceptibility to moisture loss (Yousuf, Deshi, Ozturk, & Siddiqui, 2020; Botondi, Barone, & Grasso, 2021) fresh-cut produce are especially more prone to microbial proliferation. More details related to microbial contamination issues and outbreaks were discussed in the literature review (Chapter

II). Among several foodborne pathogens, *E. coli* O157:H7, *Salmonella* spp., and *Listeria monocytogenes* are of major concern in fresh and fresh-cut produce.

Studies have reported that intrinsic and extrinsic factors such as storage temperature (Huang, Chang, & Wang, 2015; Soderqvist et al., 2017), relative humidity (Redfern & Verran, 2017), pH (Singh & Yemmireddy, 2022), moisture content (Ly, Parreira, & Farber, 2019), nutrients released from the cut surface (Heaton & Jones, 2008; Olaimat & Holley, 2012), commodity type (Huang, Luo, Zhou, Zheng, & Nou, 2019; Marik, Zuchel, Schaffner, & Strawn, 2020), and natural microbiota (Jackson, Stone, & Tyler, 2015; Cabrera-Díaz et al., 2022) significantly affect growth, survival, and death kinetics of foodborne bacterial pathogens on fresh-cut produce. A more specific discussion on the effect of these individual factors on microbial proliferation in fresh-cut produce was presented in Chapter II. Moreover, the number of post-harvest processing and storage interventions such as treatment with chemical sanitizers, e.g., sodium hypochlorite, peroxyacetic acid, hydrogen peroxide, and organic acids; and physical methods of treatment such as thermal (heat, steam) and non-thermal (ionizing and non-ionizing radiation, high-pressure processing, plasma) were commonly followed on a case-by-case basis to reduce the risk.

A careful analysis of past research work on fresh-cut produce reveals two important aspects: (i). Evaluation of microbial growth and survival kinetics using cells that are grown under ideal laboratory conditions; (ii). Development, validation, and verification of interventions based on perceived kinetics data from an earlier point. These scenarios are far from real-life conditions where microbes are constantly exposed to various environmental stresses such as stable and dynamic temperatures, relative humidity, pH, desiccation, salt stress, etc. These

stressors either increase or decrease the susceptibility of respective organisms. For example, Harrand et al (2019, 2022) reported that pre-growth conditions such as 37 °C, 21 °C, low pH 5, and high salt had a significant effect on reductions in *S. enterica* and *E. coli* population on cut produce (lettuce, cantaloupe, and tomato). Similarly, Singh & Yemmireddy (2022) reported that pre-growth environmental stresses such as low pH 5, high salinity, and desiccation exert different effects depending upon the type of pathogen, where stressed cells of *Salmonella* showed the highest sensitivity, while *Listeria* showed the least sensitivity to tested chemical treatments. Furthermore, bacteria have evolved to acquire a range of adaptive mechanisms to adjust to different environmental conditions. Thakur, Asrani, & Patial (2018) explained possible mechanisms of adaptations by differential gene expression in pathogens under adverse environmental conditions. The presence of transcription factors, such as sigma factors are responsible for the transcription of genes involved in resistance to diverse stresses (Wu, Yuk, Liu, & Ding, 2022). A study reported that *Salmonella* Enteritidis upregulates the genes involved in resistance to heat (rpoH, uspB, and htrA), cold (cspA, cspC, and csdA), acid (SEN1564A and cfa), and salt (proP, proV, and osmW) (Ye et al., 2019).

Thus, any work focusing on the effect of pre-growth environmental conditions on the subsequent microbial survival kinetics in fresh-cut produce provides valuable data to better estimate the risk and further develop risk reduction strategies. We chose *L. monocytogenes* and *Salmonella enterica* as model organisms to test against fresh-cut mixed salad. Therefore, the objectives of my thesis research were (1) to determine the survival and growth kinetics of pre-growth temperature-adapted *Listeria monocytogenes* and *Salmonella enterica* on the fresh-cut

produce under storage at 4°C and 80±2% RH, (2) effect of pre-growth environmental stresses on the response of *L. monocytogenes* to the different light treatments.

The present thesis was organized into a total of five chapters. The first chapter is an Introduction. The second chapter presents theory and literature related to topics in discussion and highlights knowledge gaps. The third chapter discusses the effect of different pre-growth temperatures on the survival and growth kinetics of *Salmonella enterica* and *Listeria monocytogenes* in fresh-cut salad during refrigerated storage. The fourth chapter presents the effect of pre-growth environmental stresses on the *L. monocytogenes* response to various antimicrobial light treatments. The fifth chapter summarizes and draws conclusions from this research work.

CHAPTER II

LITERATURE REVIEW

Fresh-cut produce

Fresh produce consumption has spiked substantially over the past few decades due to its benefits for maintaining health and meeting daily nutritional requirements. Along with the health perks, buyers are drawn to ready-to-eat (RTE) items since they are readily available, fresh, and easy to incorporate into a daily diet (Almenar, 2020). In contrast to whole, intact food, fresh-cut produce is defined as raw harvest that has undergone minimum processing, such as washing, cutting, and packaging before reaching the consumer (Botondi, Barone, & Grasso, 2021). The traditional processing of fresh fruits and vegetables involves series of procedural steps that are crucial in preparing these nutritious delights for consumption such as washing, sanitizing, peeling, shredding, cutting, or dicing, drying, and packaging (Raffo & Paoletti, 2022). According to Jideani, Anyasi, Mchau, Udoro, & Onipe (2017), washing is an effective physical method for removing dirt, pesticide residues, and microorganisms from produce surfaces. In the fresh-cut produce industry, this step is accomplished by transferring the product to the water tank on belts, either with or without a disinfectant (Raffo & Paoletti, 2022). Fresh produce's outer, inedible layer is removed during the first stage of processing by peeling. Depending on the type of produce, it can be done manually (with knives), mechanically (using abrasive tools), chemically (dipping produce in an alkaline solution to dissolve cell wall polysaccharides), enzymatically (using an enzyme solution to digest the pectin, cellulose, and hemicellulose in the cell wall), or

thermally (using high heat to cause loss of rigidity and cell wall substances). Before packaging, fresh produce is reduced in size by being cut, diced, or shred into small pieces. The shelf-life of cut produce while being stored is significantly impacted by the sharpness and shape of the knife (Gil & Allende, 2012; Li et al., 2017). Freshly cut produce is dried with forced air or air-bed conveyers before packaging to remove the moisture from the cut produce which could possibly accelerate microbial growth and spoilage (Gil, Gómez-López, Hung, & Allende, 2015). Freshly cut fruits and vegetables are packaged thereafter and made ready to eat (RTE), saving consumers time. Modified atmosphere packaging (MAP), the most popular type of packaging which delays ripening, extends shelf life, and inhibits growth of spoilage pathogens (Zhang, Meng, Bhandari, Fang, & Chen, 2015).

When fresh produce is processed, the surface tissues are removed, exposing the cytoplasm, a richer source of nutrients than intact produce to microbes (Barry-Ryan, Pacussi, & O'Brien, 2000). Accelerated metabolism, enzymatic activity, moisture loss, and other factors are blamed for this increased deterioration (Francis et al., 2012). Due to the increased surface area-volume ratio introduced by cutting techniques, fresh-cut produce tissues risk losing moisture at a higher rate than intact produce (Giannakourou & Tsironi, 2021). Processing that stimulates biochemical and physiological changes in the produce, also increased its susceptibility to microbial attack (De Corato, 2019). Moreover, elimination of the decontamination step during minimal processing, leads to increase this concern even more and puts human health at risk (Choi, Norwood, Seo, Sirsat, & Neal, 2016). Produce that is RTE or minimally processed confronts two main challenges: first, preserving its freshness without compromising its visual or nutritional value; second, improving the quality of contamination-free fresh produce to fulfill consumer demands. This review leads us to better understand the risk profiles of fresh-cut and RTE packaged

products to combat the linked multistate foodborne outbreaks. In addition, conclusions of different factors involved in survival kinetics of foodborne pathogens directing towards the future considerations.

History of outbreaks

The production of vegetables in the US was estimated to be 19 million tons with global market value of 304 billion U.S. dollars in the year 2021 (Statista, 2023). The increase in consumer demand for ready-to-eat, minimally processed fresh produce leads to large scale production and distribution to the market through supply chains where the risk of illness outbreaks becomes more prominent (Callejón et al., 2015; Alegbeleye, Singleton, & Sant'Ana, 2018; López-Gálvez, Gómez, Artés, Artés-Hernández, & Aguayo, 2021). The contamination can occur at any time from farm to fork, for instance, contaminated soil, irrigation water, manure, pest infestation, processing equipment, irregular conditions during transportation and storage, and improper handling practices (Wadamori, Gooneratne, & Hussain, 2017; Machado-Moreira, Richards, Brennan, Abram, & Burgess, 2019; Possas & Pérez-Rodríguez, 2022). Major foodborne pathogens that cause serious illness in the population including *Salmonella*, *Listeria*, *E. coli*, *Cyclospora*, *Campylobacter*, Hepatitis A, Norovirus and *Cronobacter*. Approximately 48 million illnesses, 128,000 hospitalizations, and 3,000 deaths have been reported per year according to CDC (CDC, 2022). Fresh cut produce is more prone to colonization by enteric pathogens, such as *Salmonella enterica*, *Escherichia coli*, and *Listeria monocytogenes* (Koukkidis, Haigh, Allcock, Jordan, & Freestone, 2017) due to their high moisture content and perishable nature. FAO estimated that 1.3 billion tons of food per year is lost to spoilage due to biotic and abiotic factors (Cichello, 2015). It has been estimated that about 25% of all globally

produced foods is lost due to microbial contamination (Bondi, Messi, Halami, Papadopoulou, & de Niederhausern, 2014). Animal products including milk, eggs, poultry, beef, and others are most often linked with foodborne outbreaks in the history (Gourama, 2020). However, cases of foodborne illness exclusively due to fresh produce have increased noticeably in past a few years. (Vojkovska et al., 2017). Table 1 enlists the fresh produce related foodborne outbreaks in past decade.

Whole produce vs cut produce: Risk profile

When it comes to food safety, whole and chopped produce have different risk characteristics. Contamination can occur during fresh produce cultivation, harvest, preparation, distribution chains, delivery to stores, and even at the final step in the consumers' kitchen (Machado-Moreira, Richards, Brennan, Abram, & Burgess, 2019). Major sources for the for bacterial contamination during pre-harvest cultivation are farm waste, contaminated soil and irrigation water, and domestic and wild livestock (Esmael et al., 2023). Due to its natural barriers such as fruit skin or outer layer, whole produce often has a lower chance of infection. Produce that has been cut open revealing interior flesh that offer these bacteria to thrive and flourish more than whole produce (Iñiguez-Moreno, Ragazzo-Sánchez, & Calderón-Santoyo, 2021). Once the fresh whole produce that has undergone processing, cross-contamination could be evident and possibly occurs due to unhygienic practices followed by workers (Castro-Ibáñez, Gil, & Allende, 2017). Therefore, it is essential to handle cut produce properly, including washing and storing at an appropriate temperature in order to mitigate the chance of developing a foodborne illness, also maintaining its texture, flavor, aroma and nutritive value. However, bacteria internalized in stomata or cut surfaces, as well as those located in hydrophobic areas, cavities, and rough

surfaces of leafy greens, are likely to survive sanitizer application (Zhou, Feng, & Luo, 2009). For example, cantaloupe rind surfaces with rough ridges could foster particularly strong microbial adhesion, making it challenging to clean and disinfect cantaloupe surfaces (Nyarko et al., 2016). Survival of foodborne pathogens is favored upon the physical damage such as bruising and peeling to the protective epidermal layer of the fruits and vegetables (Ward, Bedale, & Glass, 2022). On the fruit surface, bacteria utilize wide range of organic compounds like glucose and/or maltose to produce organic acids, and increases the enzymatic activity of catalase, oxidase which hasten further proteolytic and lipolytic reactions to spoil fresh products. Moreover, *Pseudomonads* are heat sensitive, hence rapidly contaminate the minimally processed fresh-cut produce (Barth, Hankinson, Zhuang, & Breidt, 2009). Rangel-Vargas et al (2018) and Huff, Boyer, Denbow, O'keefe, & Williams, (2012) revealed that all tested foodborne bacterial strains (*L. monocytogenes*, *Salmonella* Typhimurium, *S.* Typhi, *E. coli*) grew on the surface of whole and cut mangoes and jalapenos, respectively. Similarly, *L. monocytogenes* was found on the surface of whole and sliced caramel apples, intact and cut cantaloupe, romaine lettuce, celery, and cauliflower (Ward, Bedale, & Glass, 2022; Kroft et al., 2022).

Packaged fresh-cut produce: Risk profile

Freshly cut produce is packaged before exiting the processing area to retail establishments. Packaging in a controlled environment improves shelf life by delaying enzymatic breakdown to simpler molecules and metabolic processes (Nimitkeatkai, Techavuthiporn, Boonyaritthongchai, & Supapvanich, 2022). Modified atmosphere packaging (MAP) is the most used packaging with low oxygen and high carbon-dioxide (non-selective antimicrobial) gas composition to enhance the shelf-life of product by inactivating oxidizing enzymes (Wilson, Stanley, Eyles, & Ross,

2019). In order to achieve the appropriate gas composition, active MAP involves replacing gases (O_2 and CO_2) in the package. Meanwhile, passive MAP uses a specific packaging film that naturally provides the desired environment as a result of the cut-produce respiration and gas diffusion through the film (Paulsen, Barrios, & Lema, 2019). Carbon dioxide gas has been most used in modified atmosphere packaging due to its antimicrobial properties where it changes intracellular pH, nutrient uptake, and causes enzymatic inhibition. For example, its high concentration reduced the growth of pseudomonads (Oliveira et al., 2010). When not controlled, the fermentation metabolism along with growth of microorganisms is primarily responsible to produce off-flavors and reduces the shelf-life of product (Caleb et al., 2013). Multiple studies have reported the impact of efficient packaging in the reduction of microbial population in fresh produce, for instance, romaine lettuce in MAP stored at 5 °C showed decline in population of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 after 10 days (Oliviera et al., 2010). With increasing O_2 concentration and storage temperature up to 10-15 °C, the growth of pathogens highly flourishes in the fresh-cut salads comprising collard green, lettuce, spinach, escarole, cabbage, and coleslaw (Lopez-Velasco et al., 2010; Sant'Ana et al., 2012). CDC has reported several multistate outbreaks related to pre-packaged salads caused by *S. enterica, E. coli* O157:H7, and *L. monocytogenes* during year 2021 in US (CDC 2023). Alone controlled packing storage conditions will not eradicate the risk of foodborne pathogens, comprehending pathogenic growth kinetics and antibacterial interventions would lead path to establish food security.

Rate limiting factors

There are several intrinsic and extrinsic factors controlling the growth and/or survival of pathogens in fresh produce. Starting from the farm environment in-field to produce's storage

conditions i.e., retail stores for the consumers, there are many factors that regulates the growth kinetics of microorganisms including processing, storage conditions, produce's natural microbiota, environmental stresses, and phytochemicals availability as mentioned below (Figure 1).

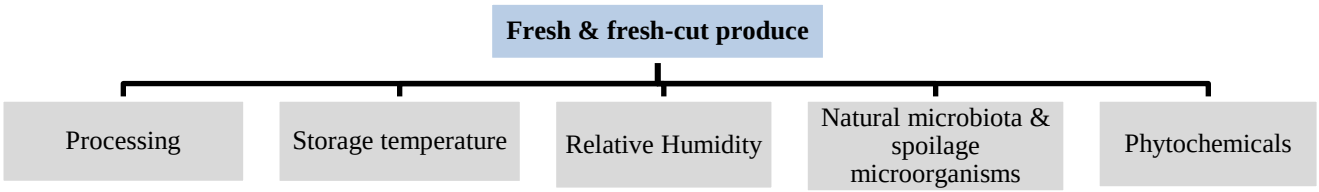

Figure 1: Factors controlling the survival of foodborne pathogens in fresh & fresh-cut produce

Effect of processing

Processing methods i.e., peeling, cutting, or shredding would expose cytoplasm and release enough nutrients for the persistence of spoilage microorganisms on the food surface (Bahram, Parvar & Lim, 2018). The freshly cut surface would likely have a higher respiration rate leading to early oxidization of cut-produce making it susceptible to microbial deterioration (Francis et al., 2012). During peeling, shredding, and cutting, an entry point for microorganisms is created which tends to increase microbial number in cut-produce when compared with intact/uncut produce (Olaimat & Holley, 2012). Further, minimal processing of fresh-cut produce accelerates microbial growth due to absence of any added preservative and antimicrobial treatments (Rashid et al., 2021). Unhygienic equipment using practices by workers during food processing may further increase pathogen persistence on fresh-cut produce (Alum, Urom, & Ben, 2016; Chatterjee & Abraham, 2018). Not only microbiologically, stressed fresh produce during processing undergoes deterioration biochemically as well such as browning, off-flavor

production, and altered taste (Yousuf, Deshi, Ozturk, & Siddiqui, 2020). Cutting tools and style imparts huge impact on the quality of cut produce such as cutting by knife caused greater injury and induction of phenol oxidation than blade cuts. Some studies have shown the difference of released nutrients among slicing and shredding the produce (Barry-Ryan & O'Beirne, 1999; Aguila et al., 2006). Packaging under controlled and modified conditions enhances the shelf-life of these cut produce available in retail stores (Sandhya, 2010).

Temperature

Temperature is one of the most important factors affecting cellular metabolic reactions and shelf-life of fresh produce (Francis et al., 2012, Kou et al., 2014). Fay et al (2023) reported that microbes proliferate with an increase in storage temperature due to acceleration of metabolic reactions and enhanced respiration of produce. Multiple studies have been conducted in this direction to elucidate the effect of varied temperature on the survival behavior of foodborne pathogens (Falah, Nadine, & Suryandono, 2015; Oliveira et al., 2015; Alegbeleye et al., 2018; Mandha, Shumoy, Matemu, & Raes, 2023). Temperature abuse is when foods are stored at temperatures exceeding the limit for safe storage (5 °C) and this abuse typically occurs during product distribution, retail display, or home storage, or transportation. For example, the population of *L. monocytogenes* on fresh-cut cantaloupes showed significant increase at different temperatures, ranging from 4 to 43 °C for 20 days (Fang, Liu, & Huang, 2013). Bardsley et al (2019) reported increased *Salmonella* and *L. monocytogenes* populations on cut cucumber stored at abusive temperature (23 °C) and survival of *Salmonella* on sliced cucumbers under refrigerated conditions. With increase in temperature, the fresh produce quality starts to degrade which attracts the growth of spoilage bacteria. Fresh-cut celery, onion, and romaine lettuce did

not support the growth of *L. monocytogenes* at 4°C, but accelerated growth was observed under storage at 10 °C or higher temperatures (Kroft et al., 2022). Similar results were observed in fresh-cut romaine lettuce stored at high temperature (Koukkidis et al., 2017; Gu et al., 2022). Wadamori et al (2017) reported that the population of *Salmonella* and *E. coli* O157:H7 in lettuce increased with the increase in temperature (5, 10, 15, 20, 25°C) from 0 to 120 h period whereas Kakiomenou, Tassou, & Nychas (1998) reported that *Salmonella* and *L. monocytogenes* failed to grow on the lettuce under storage at 4 °C. FDA advised to store the fresh fruits and vegetables at low temperature conditions (4-5 °C). Kroft et al (2022) reported that the population of *L. monocytogenes* declined on fresh-cut celery (0.75), romaine lettuce (0.92), parsley (0.94), and pineapple (3) with significant log reductions under storage at 4°C. Similar growth trend has been observed in packaged lettuce where *E. coli* O157:H7 did not grow under 5 °C refrigerated conditions but showed raised log survival by 2 log CFU/g when stored at 12°C for 8 days (Luo et al., 2010). Furthermore, type of processing and temperature shows dual effect on the microbial growth. For instance, *L. monocytogenes* did not show any growth on whole and chopped enoki mushrooms stored at 5 °C, whereas growth trend inclined by 1.89 (whole; 7d) and 1.67 (chopped, 7d) folds at 10°C, and 2.8 (whole; 7d) and 2 (chopped, 7d) folds at 25 °C (Fay et al., 2023). At abusive temperatures, both *S. enterica* and *L. monocytogenes* population increased significantly on cut melon and cantaloupe as well as juice extracts due to the high sugar content and pH, melon juices are well suited for supporting the growth of bacterial pathogens (Lee, Kim, Bang, & Yuk, 2022). Therefore, all above mentioned studies proved that temperature acts as a crucial controlling factor to facilitate or inhibit the growth of foodborne pathogens.

Relative Humidity

Relative Humidity is one of the determining factor or control strategy for the survival and/or growth of the pathogen in commodity during storage since intracellular water potential regulates enzymatic activity and hydration of produce (Marik, Zuchel, Schaffner, & Strawn, 2020; Qiu et al., 2022). Kim et al (2015) showed results stating significant correlations ($r > 0.9$, $p < 0.001$) between temperature and relative humidity and the occurrence of foodborne illness outbreaks due to *E. coli* and *Norovirus*. Many studies have shown better survival/persistence of pathogen on fresh produce stored under refrigerated temperature and high RH (> 50%) listed as Stine et al (2005); Likotrafiti et al (2013); Tian et al (2013); Castro-Ibanez et al (2015); Lopez-Galvez et al (2018). The data from Redfern et al (2017) suggested that 50% relative humidity enhances survival of *Listeria* irrespective of temperature due to less stress than higher humidity levels (85%). Contrastingly, temperature exerts more impact on the pathogenic growth on lettuce than the RH changes (Wang et al., 2012). Wang & Oh (2012) observed that low temperature (15 °C) and RH (60%), combined are capable to inhibit the growth of *E. coli* O157:H7 on spinach leaves. Similarly, Igo & Schaffner (2019) also studied the dual effect of temperature (21 °C) and RH (100%) on the growth of *E. aerogenes* increased by 7 log CFU/ /mL. Brandl & Mandrell (2002) observed decline reduction in *Salmonella* on cilantro leaves when RH decreased from 100 to 60%. The population of *L. monocytogenes* remained approximately constant on lettuce stored at 10 °C and 90% RH for 5 days. Similarly, on the intact cucumber surface, the population of pathogen remained relatively constant at 53% RH with a significant increase at 90% RH after 3 days. Similarly, *Salmonella* levels decreased by 2 log units under 60% RH when compared to 85% RH for 7 days storage period (Lopez-Galvez et al., 2018). Above-mentioned studies reflect the importance of controlled RH for survival of pathogens under cooler conditions.

Effect of natural microbiota and spoilage organisms

The diverse types of naturally occurring microorganisms are present in the fresh produce from the field to fork continuum and most likely depends on the type of produce, weather conditions at harvesting, geographical location, and further handling of products (Ramos et al., 2013; Kusstatscher et al., 2020). The microbiota of vegetables and fruits comprise of *Pseudomonas* spp., *Erwinia herbicola*, *Flavobacterium*, *Xanthomonas*, and *Enterobacter agglomerans* as well as various yeasts and molds (Qadri et al., 2015; Piombo et al., 2018; Sui et al., 2020). All the bacterial strains within natural microbiota are not pathogenic and present even at the time of consumption (Jackson, Stone, & Tyler, 2015). Lactic acid bacteria (LAB) (L*euconostoc mesenteroides* and *Lactobacillus* spp.), and other natural microbiota commonly act as antagonist to the problematic pathogens (Mohamed, Shady, Sayed-Ahmed, & Amer, 2016; Raman et al., 2022). LAB produces bacteriocins (nisin) and other metabolites (lactic acid, ethanol, carbon dioxide) inhibiting the vegetative and reproductive growth of pathogenic bacteria, hence used as natural food preservatives with or without traditional preservation routine (Barbosa, Mantovani, & Jain, 2017). LAB does not use oxygen to produce their energy, they grow well under anaerobic conditions, but can also grow in the presence of oxygen (Maresca, Zotta, & Mauriello, 2018). Interestingly, nutrients released from the cut surface of produce supports the proliferation of LAB (Jaffar, Jawan, & Chong, 2023). On the contrary, the LAB species *L. mesenteroides* could be involved in the spoilage of shredded carrot after 9-12 days storage under 10 °C (Lampert et al., 2017). Other spoilage microorganisms showed higher contamination levels of aerobic plate counts (APC) in unwashed carrot (5.57 log CFU/g), zucchini (5.69 log CFU/g), and green bell peppers (5.71 log CFU/g) (Sun, Kim, Kwak, & Yoon, 2012). Mesophilic bacteria from plate count studies typically range from 10^3 to

10^9 CFU/g. The Mesophilic aerobic bacteria (MAB) proliferated on cantaloupes inoculated with *L. monocytogenes* by 1.5 and 7 log after 7 days storage at 4 and 15 °C, respectively. These results indicated that *L. monocytogenes* proliferated on cut cantaloupes because of its physicochemical characteristics and lack of antagonistic microbiota (Gu et al., 2022).

Fungi, which include yeasts and molds, are another group of microorganisms that can spoil fresh cut fruits and vegetables at ambient temperature or MAP storage. Neutral pH 7.0 is optimal for the growth of most microorganisms. However, yeasts (pH range of 310) and molds (pH 2-11) are usually acid tolerant and are therefore associated with the deterioration of acidic foods (Alegbeleye et al., 2022). Among the yeast species, genera *Saccharomyces, Candida, and Hansenula* have been associated with spoilage of fresh cut products and juices (Kaczmarek, Avery, & Singleton, 2019). Spoilage by mold is caused by species of *Penicillium, Phytophthora, Alternaria, Botrytis, Fusarium, Cladosporium, Phoma, Trichoderma, Aspergillus, Alternaria, Rhizopus, Aureobasidium, and Colletotrichum* (Barth et al., 2009). High yeasts and molds counts have been observed in spinach, lettuce, tomato, and apple under retail storage conditions (4 °C) (Tango et al., 2018). Oliveira et al (2015) reported that yeast helps in decaying the fermented products to develop soft decay. Mycotoxin production, which has a significant influence on the quality of fresh produce and juices, is an important characteristic of some of these spoilage fungi (Ismaiel & Papenbrock, 2015).

Phytochemicals

Phytochemicals such as vitamins, polyphenols, carotenoids, glucosinolates, and anthocyanins are abundantly present in fresh produce (Manganaris, Goulas, Mellidou, & Drogoudi, 2018). Plant derived natural preservatives and antimicrobials are healthier and safer

due to their nutritional value and bioactivity (Yuan et al., 2022), hence promising to intervene. Polyphenols are one of the major secondary metabolites found in fruits, vegetables, coffee, tea and alcoholic beverages (Serra, Almeida, & Dinis, 2018). Studies have found that polyphenols rich diet is associated with the less incidence of chronic diseases (Xiao & Hogger 2015; Guo, Shan, Yang, Jiang, & Zhu, 2017; Costa et al., 2017; Chen, Zhao, Li, & Yang, 2022). The antimicrobial property of phenolics directly comes from their ability to interact and penetrate the cell membrane of organisms, further inducing coagulation of cell content, irreversibly damaging intracellular enzymes such as DNA gyrase, and cause accumulation of ATP, NADP and other cellular metabolites as ROS, ultimately led to cell death (Patel et al., 2015; Stokes, Lopatkin, Lobritz, & Collins, 2019). Interestingly, Gram-negative bacteria are more resistant to plant secondary metabolites than Gram-positive bacteria (Papadopoulou & Frazier, 2005; Wu, Qiu, Bushway, & Harper, 2008; Negi, 2012; Caillet, Côté, Sylvain, & Lacroix, 2012; Lau, Barbut, Ross, Diarra, & Balamurugan, 2019), since gram-negative bacteria has a protective lipopolysaccharides envelope outside the peptidoglycan layer which decelerate the passage of phytochemicals (La Storia et al., 2011; Konate et al., 2012). Various studies have been conducted to assess the antimicrobial activity of plant extracts, but its application to food products has not been properly discussed due to their reducing effectiveness and instability (Bouarab-Chibane, Degraeve, Ferhout, Bouajila, & Oulahal, 2018). Among fresh produce, for instance, carrots exerted inhibitory growth of *L. monocytogenes* because of phytoalexins released by carrots under pathogenic stress to suppress the growth of gram-positive bacteria and fungi (Noriega et al., 2010; Ma et al., 2015; Alegbeleye & Sant'Ana 2022). Kim et al (2022) reported that dehydrocorydaline, a phytochemical, caused carbohydrate metabolism dysfunction, alter gene expression of virulent genes, and thereby, increased the intracellular ROS in *L.*

monocytogenes, leading to the cell death. Phytochemicals can inactivate the bacteria by modulating virulence factors and disrupting cellular mechanisms after penetrating its cell membrane (Silva, Zimmer, Macedo, & Trentin, 2016).

Another significant secondary metabolite, flavonoid, showed antibacterial and bacteriostatic property by damaging the cell wall, causing leakage of cellular contents, and inhibiting protein synthesis (Cushnie & Lamb, 2011; Zhao, Chen, Zhao, & Yu, 2015; Rao et al 2018; Limwachiranon, Huang, Shi, Li, & Luo, 2018). Beermann et al (2013) studied the effect of polyphenolic extracts of apples on the growth of both Gram-positive and Gram-negative bacteria. They observed that growth reductions were approximately -35% to -15.51% compared to control samples. Papadopoulou & Frazier, (2005) reported that the bacteriostatic effect of apple pomace extract directly depends on the polyphenolic content. Similar findings were reported by Guo et al (2022) indicating the anti-microbial role of tea polyphenols. It was deduced that polyphenolic compounds enhanced the carbohydrate and other nutrients' absorption (Garcia-Ruiz et al., 2009). Lau et al (2019) studied the effect of cranberry extract (CE) on the growth of food borne pathogens, where all three *L. monocytogenes*, *E. coli 0157:H7* and *S. enteritidis* were failed to grow at different concentrations (0.5, 0.75, 1.0%) of polyphenols rich cranberry extract. They also proposed that CE, at significant levels, may inhibit the growth of food-borne pathogens and proliferate the population of beneficial bacteria. One study by Torres et al (2007) suggested that *Lactobacillus* species can survive abusive conditions such as high acidity, ethanolic and phenolic concentrations. This could be due to the high energy generating systems or membrane permeability preventing shock from phenolic cranberry extract. Also, *Lactobacillus* species may hold polyphenolic catabolic mechanisms to metabolize polyphenols and hence, increase tolerance (Lacombe et al., 2013). Lau et al (2019) supported those cranberry organic

acids may create osmotic pressure causing sub-lethal damage to bacterial cell membrane. *S. enterica* population dropped after 7 days storage at 4 °C on cantaloupe, watermelon, and radish except pineapple since cut radish hindered the bacterial growth due to its antibacterial secondary metabolites. Sant'Ana et al (2012) reported *L. monocytogenes* failed to grow on carrots due to the presence of anti-listerial compounds of the produce. Among fresh produce, red cabbage is rich source of antioxidants such as anthocyanins, glucosinolates, and sulfur compounds such as methyl methanethiosulfinate known to have inhibitory effects on the foodborne pathogens (Demirdoven, Karabıyıklı, Tokatlı, & Öncül, 2015; Alvarez, Moreira, Roura, Ayala-Zavala, & González-Aguilar, 2015; Barbieri et al., 2017; Favela-Gonsalez, Hernández-Almanza, & De la Fuente-Salcido, 2020). Wu et al (2008) and Borges, Ferreira, Saavedra, & Simões (2013) hypothesized that damaged bacterial membrane facilitates leakage of cellular contents and allow certain phenolic compounds to enter the cell and induce metabolic disruptions. Similar findings were reported by Chen, Zhao, Meng, & Yu, (2017) where they tested the effects of polyphenolic sugar beet molasses on the food borne pathogens (*S. aureus*, *S.* Typhimurium, *E. coli* and *L. monocytogenes*) and observed increased protein leakage from the bacterial cell by approximately 2-fold. Their results of SEM and TEM showed that polyphenols damaged the external structure of *S. aureus* and *E. coli* and caused leaking of cytoplasmic components. The results of SDS-PAGE indicated that the disappearance of protein bands might be due to their (polyphenols) interference with protein synthesis and controlling the gene expression. Similar results were reported by Zhao et al (2015), who suggested that lactic acid reduces protein content in bacterial cells by penetrating and destroying cell membranes. However, these phenolic compounds undergo oxidation and gets converted into quinones further causing browning to fresh produce. Principal enzyme, polyphenol-oxidase (PPO) catalyzes the reaction in wounded or cut surface of

minimally processed fruits and vegetables. Such instances initiate the signal to induces metabolic pathway responsible for increased production of phenolic compounds and consequent browning in lettuce leaves (De Corato, 2019).

Experimental conditions and limitations

Foodborne pathogens experience many unfavorable conditions throughout their life cycle, particularly during food production, processing, storage, and cooking. Many studies have mentioned the role of filamentous growth as adaptation to several stresses in *L. monocytogenes*, *E. coli*, and *Salmonella* such as starvation, pH stress, high-pressure, low water activity, low temperature, high CO_2, and antimicrobials (Jones, Stres, Rosenquist, & Hallin, 2008; Quiroz-Baez, Rojas, & Arias, 2009; Vázquez-Gutiérrez et al., 2011; Pratt, Chen, Czuprynski, Wong, & Kaspar, 2012; Vail, McMullen, & Jones, 2012; Visvalingam, Hernandez-Doria, & Holley, 2012). These stresses stimulate responses in bacteria such as production of proteins that repair damage, maintain cell homeostasis, or removal of the stress agent (Begley & Hill, 2015). Heat shock proteins (HSP)/cold shock proteins (CSP) and acid shock proteins (ASPs) are induced in bacterial cells when subjected to high/low temperatures, and low pH respectively (Arsène, Tomoyasu, & Bukau, 2000; Wouters et al. 2000; Alvarez-Ordonez et al., 2012). Haberbeck et al (2017) defined cross-protection as adaptation of bacteria to stress conditions by eliminating via survival or proliferation under such unfavorable conditions. Beales (2004) reported that low temperatures for *L. monocytogenes* growth increase the degree of unsaturated fatty acids enhancing membrane fluidity. *L. monocytogenes* could grow even at low temperatures as it has cryoprotectants such as glycine betaine and carnitine, and stress sigma factor σ^B to adapt cold stress (Becker, Evans, Hutkins, & Benson, 2000; Angelidis & Smith,

2003). *L. monocytogenes* showed better adaptations under desiccation and acid stress than *Salmonella* and *E. coli* due to higher resistance power (Harrand et al., 2021). Bacteria, either in food with acidic additives (organic acids) or within human gut, often encounter low pH condition grows stress tolerance for its survival. Gene expression profiling showed that preadaptation to cold stress has resulted in increased gene expression related to acid stress and oxidative stress to protect against damage (Begley & Hill, 2015). Many studies have focused on the ability of foodborne pathogens in biofilm formation under unfavorable conditions, mainly in food manufacturing and processing facilities (Xu, Lee, & Ahn, 2011, Bridier et al 2015, Giaouris et al., 2015, Colagiorgi et al., 2017, Galie et al., 2018, Liu et al 2023). This occurrence is of concern *since L. monocytogenes* is commonly isolated from food processing environments (Gandhi & Chikindas 2007). Vasseur, Baverel, Hébraud, & Labadie (1999) reported the effect of different stress conditions on the five strains of *L. monocytogenes* and concluded doubling of generation time (salt stress), increased lag phase (alkalinity stress), increased growth rates with longer lag times (acid and heat stress), and no visible effect on cells under cold stress. Conclusively, major foodborne bacteria possess abilities to resist various environmental stresses for longer persistence which needs to be explored further under different conditions.

Prospects and challenges

Growing demand for fresh produce due to increasing health consciousness creates opportunities for research on the safety of fresh produce and need for international collaboration to ensure food safety standards are new prospects in this field. Ample literature is present aiming

to determine the survival kinetics of foodborne pathogens grown under normal laboratory conditions (37 °C) on the fresh produce where different kinds of stresses (temperature, desiccation, pH, sanitizers) are exerted on them post-growth. However, in real life, these pathogens undergo various stresses pre-growth and even during the growth phase such as pathogens proliferating under transportation and storage conditions from farm to fork chain. To protect public health and improve food safety, it is necessary to understand the growth behavior of stressed cells of foodborne pathogens in all types of ready-to-eat salads that are commonly available and consumed, to accurately present their survival state. Several characteristics of leafy green pathogenic outbreaks including their short shelf life (12–16 days) (FDA, 2010), delay in identifying outbreaks (22 days), the short duration of most outbreaks (21 days), and difficulty in identifying the source of contamination in fresh produce due to the complex supply chains and diverse production systems makes them challenging to implement timely interventions to reduce illness. All these reasons limit the opportunities for investigators to scrutinize the disease on time. Also, intrinsic factors such as phytochemicals have been shown to check the growth behavior of pathogens under certain conditions. Little information is available on the effect of commodity types on the growth and survival of different foodborne pathogens which could be explored more in the future. Development of new and improved production techniques for fresh produce which may further reduce the incidence of foodborne pathogens in crops is needed. Since UV light has carcinogenic effects on the workers, there is a lack of efficient alternative methods to reduce the bacterial population on surfaces of fresh produce without changing the physical and chemical properties of food components. Such limitations lead to the prospective use of visible light (400-500 nm) as effective antimicrobial agents on which limited literature is available.

Knowledge gaps & Conclusions

This review addresses the previous studies focusing on the fresh produce and different intrinsic and extrinsic factors affecting the growth/survival kinetics of the foodborne pathogens in the fresh produce. The transmission of pathogens to humans and the result or severity of a disease are largely influenced by these factors, either positive or negative. In order to reduce the risk of foodborne diseases in fresh produce, there is a need for improved hygiene practices, enhanced education for growers and consumers, implementation of appropriate storage and cooling regimes. The presence of diverse and healthy microbial communities on fresh produce can promote food safety by minimizing the occurrence of pathogenic organisms as certain beneficial bacteria and fungi can outcompete pathogens for resources from the produce surface, thus reducing their growth. Overall, it is crucial to pay close attention to the effects of storage conditions on the survival of foodborne pathogens in fresh produce to ensure the safety of fruits and vegetables in a manner that reduces the risk of foodborne illness and contamination. There is a critical need to develop simple and rapid assays to detect specific spoilage bacteria and to better understand the links between spoilage microbial populations and spoilage of whole and fresh-cut foods. Further studies should be carried out to provide processors with appropriate technology to safeguard safety and maintain the nutritional and sensory characteristics of such products as a growing number of consumers are interested. To understand the behavior of bacteria persistence and tolerance linked to food associated stress, better understanding at molecular level is needed. Lastly, the effect of pre-growth temperature conditions on the survival of foodborne pathogens is clearly worthy of further investigation.

Objectives

1. Effect of different pre-growth temperatures on the survival and growth kinetics of *Salmonella* and *Listeria monocytogenes* in fresh-cut salad during refrigerated storage.
2. Effect of pre-growth environmental conditions on the *L. monocytogenes* response to various light treatments.

Table 1: List of foodborne outbreaks in fresh produce in US during 2013-2023.

Commodity	**Pathogen**	**Characteristics**	**Active year**	**Reference**
Ready-to-eat salads	*E. coli* O157:H7	Fresh cut	2013	Mikhail et al., 2018
Cucumbers	*Salmonella* Saint Paul	Whole	2013	CDC 2013
Peach	*L. monocytogenes*	Whole	2014	Jackson et al., 2015
Lettuce	*L. monocytogenes*	Packaged salad	2015	Self et al., 2016
Cucumbers	*S. enterica* Poona	Whole	2016	CDC 2016
Cantaloupes	*Salmonella* sp.	Whole and cut	2017	CDC 2017
Melon	*Salmonella* sp.	Packaged pre-cut	2018	CDC 2018
Papaya	*Salmonella* Uganda	Whole	2019	CDC
Romaine lettuce	*E. coli* O157:H7	Whole	2019	FDA 2020
Onions	*Salmonella* Newport	Whole	2020	McCormic et al., 2022
Peaches	*Salmonella* Enteritidis	Whole	2020	FDA 2020
Leafy greens	*E. coli* O157:H7	Pre-mixed	2020	Lacombe et al., 2022
Prepackaged Salads	*Salmonella* Typhimurium	Whole, pre-mixed	2021	McClure et al., 2021
Baby spinach	*E. coli* O157:H7	Whole	2021	FDA 2021
Dole Packaged Salads	*Listeria monocytogenes*	Fresh cut	2021	CDC 2022
Fresh Express Packaged Salads	*Listeria monocytogenes*	Cut	2021	FDA 2021
Power Greens Packaged Salads	*E. coli* O157:H7	Cut	2021	CDC 2022
Enoki Mushrooms	*Listeria monocytogenes*	Whole	2022	FDA 2022
Leafy Greens	*Listeria monocytogenes*	Fresh cut	2023	CDC 2023

CHAPTER III

EFFECT OF DIFFERENT PRE-GROWTH TEMPERATURES ON THE SURVIVAL AND GROWTH KINETICS OF *SALMONELLA* AND *L. MONOCYTOGENES* IN FRESH-CUT SALAD DURING REFRIGERATED STORAGE

Abstract

The effect of the growth temperature of bacterial cultures on their subsequent survival kinetics in fresh-cut produce during refrigerated storage was investigated in this study. Three-strain cocktails of *L. monocytogenes* and *Salmonella enterica*, cultured at different growth temperatures (4, 21, and 37 ºC) were inoculated on fresh-cut mixed salad and on its individual produce items. The samples were stored at 4°C and 80±2 % relative humidity for up to 72 h and growth or survival was determined at regular intervals. The results indicate that depending upon the type of pathogen tested, the pre-growth temperature(s) and the type of produce showed a significant ($p \leq 0.05$) effect on the survival kinetics during the storage. Among the tested produce, mixed salad showed the highest reduction of *L. monocytogenes* pre-grown at 37 ºC (1.33) followed by red cabbage (0.56), iceberg lettuce (0.52), and carrot (-0.62 log CFU/g), after 72 h respectively. In the case of *Salmonella*, carrot showed the highest reduction (1.07, 37 ºC) followed by mixed salad (0.78, 37 ºC), cabbage (0.76, 21 ºC), and lettuce (0.65 log CFU/g, 4 ºC), respectively. Baranyi-Roberts model better fitted the data in predicting the kinetics. These findings indicate that appropriate selection of pre-growth environmental conditions is critical to better understand the kinetics of foodborne pathogens.

Keywords: Fresh-cut salad, temperature stress, foodborne pathogens, storage, survival kinetics

Introduction

Leafy greens are a vital source of vitamins, minerals, dietary fiber, and phytonutrients and are attributed to lowering the risk of several chronic diseases such as diabetes, obesity, cardiovascular disease, hypertension, and cancer (Boeing et al., 2012; Zhang et al., 2015; Aune, 2019). In the recent past, demand for fresh, convenient, preservative-free, minimally processed, and health-promoting foods such as fresh-cut fruits and ready-to-eat (RTE) mixed salads has increased (Miranda et al., 2016; Miceli & Settanni, 2019; Almenar, 2020). However, fresh-cut produce is prone to rapid deterioration due to moisture loss, accelerated enzymatic activity, and microbial proliferation (Francis et al., 2012; Yousuf, Deshi, Ozturk, & Siddiqui, 2020; Botondi, Barone, & Grasso, 2021). Moreover, leafy greens have been implicated in several foodborne illness outbreaks (Callejon et al., 2015; Carstens, Salazar, & Darkoh, 2019; Irvin et al., 2021). Foodborne bacterial pathogens such as *Listeria monocytogenes* (Zhu, Gooneratne, & Hussain, 2017; Alegbeleye & Sant'Ana, 2022; Gu et al., 2022) and *Salmonella* enterica (Wadamori, Gooneratne, & Hussain, 2017, Carstens et al., 2019; Glaize, Young, Harden, Gutierrez-Rodriguez, & Thakur, 2021) are associated with multiple illness outbreaks in fresh and fresh-cut produce.

L. monocytogenes is of particular concern in RTE salad mix due to its ability to grow in aerobic or anaerobic environments, as well as at refrigeration temperatures (Pereira, Alves, Ferreira, & Teixeira, 2018; Smith et al., 2018; Ly, Parreira, & Farber, 2019). In 2021, a multi-state outbreak of packaged salads was attributed to contamination with *L. monocytogenes* (CDC,

2021). Studies revealed that nutrients released from exposed cytoplasm of cut fresh produce enable the proliferation and growth of foodborne pathogens (Qadri, Yousuf, & Srivastava, 2015; Jideani, Anyasi, Mchau, Udoro, & Onipe, 2017). Many studies have determined the survival kinetics of foodborne pathogens in fresh-cut salads and produce during storage. For example, *L. monocytogenes* (*Lm*) showed a reduction of 3 (Arabic salad at 4 ºC), 2.3 (tahini salad at 4 ºC), 3.3 (basil at 21 ºC), 2.4 (cilantro at 21 ºC), 2.6 (dill at 21 ºC), and 3.2 (parsley at 21 ºC) log CFU/g during storage (Olaimat et al., 2021; Bardsley, Boyer, Rideout, & Strawn, 2019). While, few studies reported an increase in *L. monocytogenes* growth by 1.5 (at 8 ˚C) and 2.3 (at 12 ˚C) log CFU/g in iceberg lettuce (Tucci et al., 2019); 3.02 log CFU/g in salad mix of lettuce, cucumber, tomato, carrot, and red cabbage (Gyorgy, Laslo, & Csató, 2020); 1.85 (arugula), 0.88 (spinach), 0.51 (green salad), 0.49 (escarole), 0.30 (collard green), 0.21 (watercress), and -3.61 (carrots) log CFU/g at 7˚C (Sant'Ana, Barbosa, Destro, Landgraf, & Franco, 2012); 1.4 (broccoli) and 1.6 (cauliflower) log CFU/g at 4 ºC (Pinton, Bardsley, Marik, Boyer, & Strawn, 2020). A study by Waitt, Kuhn, Welbaum, & Ponder (2014) reported a non-significant decrease in the population of *Salmonella* on lettuce roots and leaves at 4 °C while temperature abuse at 12 °C increased *Salmonella* levels. Lee, Han, Yoon, & Lee, (2022) reported that populations of *E. coli* O157:H7 (2.2 log CFU/mL), *Salmonella*, and *L. monocytogenes* (2 log CFU/mL) increased rapidly in red cabbage juice at 10 °C up to 72 h storage period. Huang, Luo, Zhou, Zheng, & Nou (2019) reported that except in pineapple, a decrease in *S.* enterica levels on cantaloupe, watermelon, and radish for 7 days of storage at 4 ºC. López-Gálvez, Gil, & Allende (2018) showed that *Salmonella* survived better on lettuce at 85 % RH than at 60 % RH. Singh & Yemmireddy (2022) reported an increase in *Salmonella* levels from 4.56 to 6.75 log CFU/g on fresh-cut papaya after 7 days of storage at 21 °C and 90% RH. These studies indicate that

survival and growth kinetics of fresh and fresh-cut produce will be dependent upon various factors such as type of pathogen, and type of produce (Lokerse, Maslowska-Corker, Van de Wardt, & Wijtzes, 2016; Jechalke et al., 2019; Jacob & Melotto, 2020), and storage conditions such as time, temperature, and RH.

Furthermore, studies reported that the presence of anti-listerial phytoalexins in carrots inhibited the growth of *L. monocytogenes* (Ma et al., 2015; Zeiglar, Kent, Stephan, & Guldimann, 2019; Alegbeleye & Sant'Ana, 2022). Huang et al., (2019) concluded that cut radish hindered bacterial growth due to its antibacterial properties whereas its juice supported the growth of *Salmonella* and *L. monocytogenes* due to the longer lag phase. It should be noted that most studies focused on determining the growth kinetics of foodborne pathogens which were cultured at ideal laboratory growth conditions. Limited information exists on the effect of pre-growth stresses on the survival kinetics of foodborne pathogens on real food matrices. Harrand, Kovac, Carroll, Guariglia-Oropeza, Kent, & Wiedmann (2019) observed the effect of pre-growth conditions on the growth of *Listeria and E. coli* on select fresh fruits and vegetables. Similarly, *L. monocytogenes* when subjected to pre-growth stresses showed less reductions upon exposure to different chemical treatments than *S. enterica* and *E. coli* O157:H7 (Singh & Yemmireddy, 2022). However, the effect of different pre-growth conditions on the fate of *L. monocytogenes* and *Salmonella* in the fresh-cut salad to represent real-world scenarios is not well understood. Thus, the main objective of this study was to determine the effect of pre-growth temperature and type of produce on the growth, survival, and/or death kinetics of *L. monocytogenes* and *Salmonella* during simulated retail storage.

Materials and Methods

Selection of bacterial strains

A three-strain cocktail of *L. monocytogenes* (i. F8027, serotype 4b, celery isolate; ii. 101M, serotype 4b, beef-associated outbreak isolate; and iii. F8385, serotype 1/2b, carrot isolate) and a three-strain cocktail of nalidixic acid adopted *Salmonella* enterica (i. Newport 11590 K, beef isolate; ii. Poona A3279, cantaloupe associated outbreak; iii. St. Paul E20081236, jalapeno outbreak) were used as test pathogens. All the *Salmonella* strains were modified for antibiotic resistance using 0.05 g L^{-1} nalidixic acid (NA; Fisher Scientific, NJ, USA) using a continuous stepwise exposure (Parnell, Harris, & Suslow, 2005). The stocks of all the strains were stored at -80 ˚C containing tryptic soy broth (TSB, Hardy diagnostics, CA, USA) and 25% glycerol (*wt/wt*). The frozen stocks of selected strains were thawed at room temperature in a biosafety cabinet and activated by transferring a loopful of inoculum into 10 mL tryptic soy broth with 0.1% yeast extract (TSBY) for *L. monocytogenes* and 10 mL of tryptic soy broth with nalidixic acid (TSBN). These cultures were then grown under different temperature conditions as discussed below.

Growing bacterial cultures and inoculum preparation

Individual strains of selected bacteria were grown under three different temperatures. Briefly, (i) strains of *S.* enterica and *L. monocytogenes* were grown in TSBN and TSBY, respectively at 37 ºC and 200 rpm in a shaker incubator for up to 18 h. This condition is referred to as a common laboratory approach followed in many studies, (ii) cells were grown at 21 ºC reflecting growth at room temperature, and (iii) cells were grown at 37 ºC for 8 h and then adapted to cold stress at 4 ºC for up to 40 h to achieve desired working concentration. Afterward, an equal volume (5 mL) of each individual strain in each category was mixed to prepare three-

strain cocktails and were harvested by centrifuging at 4000 X g for 10 min (5920R, Eppendorf^{TM}, Hamburg, Germany). The resultant pellet(s) were re-suspended in 10 mL of 0.1% buffered peptone water (BPW, Hardy Diagnostics, CA, USA), and serial dilutions were performed to achieve a working concentration of 10^6 CFU/mL. Cell concentrations were confirmed by plating 100 µL portions of appropriate serial dilutions on selective media Oxford agar (*Listeria*) and xylose lysine deoxycholate supplemented with 0.05 g L^{-1} nalidixic acid agar (XLDN; *Salmonella*) plates and incubation at 37ºC for 24 h.

Sample preparation, inoculation, and storage

Fresh iceberg lettuce (*Lactuca sativa* cv capitata), red cabbage (*Brassica oleracea* cv capitata f. rubra), and carrot (*Daucus carota*) were purchased from the local retail store (McAllen, TX) and stored at 4˚C for use in experiments within 24 h. After removing the outer 2-3 layers of lettuce and cabbage, all the vegetables were thoroughly rinsed with deionized water and air-dried in a biosafety cabinet at room temperature. The dried lettuce and cabbage were aseptically chopped to a desirable size (5 cm length x 2 cm width) and the carrots were grated as shown in Fig 1. A 50 g sample(s) weighed in stomacher bags (Whirl-Pak™, WI, USA), and an aliquot of 5 mL of inoculum was added to each individual bag. The bags were thoroughly mixed by shaking and gentle massaging for about 2 min to achieve a uniform distribution of the inoculum on each sample. The individual batches of inoculated samples were pooled together in a sterile tray (250 g total sample size for each produce item) and mixed again using a sterile spatula. For the mixed salad samples, 70: 20: 10 (wt %) of iceberg lettuce, red cabbage, and carrot were mixed and then followed the inoculation procedure as described above. All the samples were air-dried in a biosafety cabinet for 1 h and portions of 25 g weighed in 20 Oz clam shell boxes (Dart, MI, USA) and then stored in an environmental chamber (Forma 3900 Series,

Thermo Scientific, MA, USA) at 4˚C and 80 ± 2% RH (Fig. 1) to simulate retail storage conditions. Appropriate control samples inoculated with sterile deionized water were also included. Samples were collected at regular intervals (0, 12, 24, 48, and 72 h) and the viability of test pathogens (both on treatment and control samples) and spoilage organisms (only on control samples) were enumerated.

Enumeration

At each sampling time, an aliquot of samples (10 g) was collected from the clamshell containers and transferred to Whirl-pak™ bags containing 90 mL (1:9 w/v) of 0.1% BPW. These samples were homogenized using a stomacher (Model 400 Seward™, NE, USA) for 2 min at 200 rpm. Appropriate serial dilutions of the samples were prepared in BPW and plated on Oxford agar (for *L. monocytogenes*) and XLDN (for *Salmonella*) in duplicates. The plates were incubated at 37°C for 24 ± 2 h, and the surviving cells were reported as log CFU/g. The rate of spoilage during storage caused by yeast and mold, aerobic plate count (APC) was determined using 3M Petrifilms (3M™, St. Paul, MN) by following the manufacturer's instructions.

Modeling survival kinetics

ComBase predictive models were assessed to determine the models that best fit the experimental data using the Online DMFit tool. The data was analyzed using Baranyi and Roberts (1994), and Linear models to determine the predictive power of these models.

Statistical analysis

All the experiments were conducted in triplicates and duplicate samples were included in each replicated experiment. The CFU data were transformed into log CFU/g and arranged to conduct statistical analysis by comparing the results within the same commodity and across

different commodities during the storage for different pre-growth temperature conditions. Data were analyzed by the analysis of variance (ANOVA) procedure using SPSS™ (Version 28, IBM®). Tukey's multiple comparison test was performed to determine the mean differences. All the tests were performed with a 0.05 level of significance.

Results and discussion

Effect of pre-growth temperature and type of commodity on survival kinetics of *L. monocytogenes*

Figure 2 shows the survival kinetics of *L. monocytogenes* that were pre-cultured at 37 (Figure 2a), 21 (Figure 2b), and 4 (Figure 2c) ºC, respectively, and subsequently inoculated on different types of produce during simulated retail storage at 5 ºC and 80±2 % RH for up to 72 h. Under the tested conditions, the results indicate that the type of produce and the culture growth temperatures have a significant ($P \leq 0.05$) effect on the survival kinetics of *L. monocytogenes*. For example, when *L. monocytogenes* were cultured at normal laboratory growth conditions of 37ºC in nutrient-rich media, depending upon the type of produce a slight increase (carrot, $P > 0.05$) or decrease (lettuce, red cabbage, and mixed salad; $P \leq 0.05$) in the levels were observed at the end of 72 h storage (Figure 2a). Highest reduction of 1.33 log CFU/g was observed in case of mixed fresh-cut salad followed by 0.56 (red cabbage), and 0.52 (iceberg lettuce) log CFU/g, respectively. However, no significant ($P > 0.05$) change in the levels of *L. monocytogenes* was observed in the case of grated carrot. At the end of 72 h refrigerated storage, survival of *L. monocytogenes* on the mixed salad was significantly ($P \leq 0.05$) lower when compared to red cabbage or iceberg lettuce and grated carrot alone possibly due to cooperative effect of different

metabolites present in individual commodities. These findings potentially can be attributed to two main factors: (i) the ability of *L. monocytogenes* to survive at refrigerated temperatures, (ii) the physical condition and/or chemical properties of tested produce, and their interaction with *L. monocytogenes* at the tested storage conditions. It is understandable as a psychotropic organism; *L. monocytogenes* can be able to survive at low temperatures, but the rate of survival was dependent upon the type of produce and storage time and temperature. Similar results of *L. monocytogenes* were observed when packaged vegetables were stored at 4 ºC (Francis et al., 2012). Whereas Carrasco et al., (2008) reported a 3 log CFU/g increase in the *L. monocytogenes* population in RTE lettuce after storage at 13°C for 7 days. This shows that time and temperature are critical factors either increasing, maintaining, or decreasing the levels of *L. monocytogenes* in fresh produce. In our study, relative humidity was also included as an additional factor to best simulate the survival kinetics of *L. monocytogenes* in tested produce during short-term retail or consumer storage.

Studies also reported that minimally processed fresh-cut or grated vegetables facilitate the attachment and further survival of pathogens due to the release of nutrients to nourish the attached bacteria and aids proliferation (Patel & Sharma, 2010; Jideani et al., 2017; Smith et al., 2018) or death due to release of antimicrobial compounds such as phytochemicals and presence of natural microbiota (Salazar et al., 2017; Gu et al., 2018; Hellstrom, Granato, & Mattila, 2020). For example, studies reported that cut red cabbage at low temperatures did not support the growth of *L. monocytogenes* (Salazar et al., 2022) and *Salmonella* (Alegbeleye & Sant'Ana, 2022), respectively. Red cabbage is a rich source of antioxidants such as anthocyanins, glucosinolates, and sulfur compounds such as methyl methanethiosulfinate known to have inhibitory effects on foodborne pathogens (Demirdoven, Karabıyıklı, Tokatlı, & Öncül, 2015;

Alvarez, Moreira, Roura, Ayala-Zavala, & González-Aguilar, 2015; Barbieri et al., 2017; Favela-Gonsalez, Hernández-Almanza, & De la Fuente-Salcido, 2020). Similarly, the inhibitory effect of grated or cut carrots on *L. monocytogenes* (Ziegler et al., 2019; Lee et al., 2022) and *S.* Typhimurium (Viswanathan & Kaur, 2001) was reported. Antimicrobial properties of phytoalexin such as 6-methoxymellein present in carrots potentially attributed to these reductions (Viswanathan & Kaur, 2001). In the current study, no significant difference in the levels of *L. monocytogenes* was observed between iceberg lettuce and red cabbage (Figure 2a). This can be attributed to limited or no exposure to available phytochemicals in red cabbage and grated carrot within the tested storage period to potentially exert significant reduction as noticed in previous studies.

Most previous studies used bacterial pathogens that were cultured at ideal laboratory growth conditions of 37 ºC in nutrient-rich media. However, increasing evidence shows that pre-growth environmental conditions affect the tolerance, growth, and survival kinetics of pathogens (Harrand et al., 2019, 2021; Singh & Yemmireddy, 2022). Thus, we investigated the survival kinetics of *L. monocytogenes* when subjected to different pre-growth temperatures. When using the *L. monocytogenes* cells that were pre-cultured at 21 ºC, irrespective of the type of produce no significant ($P>0.05$) change in the levels were observed during the storage (Figure 2b). Non-significant fluctuations across all the produce types were noticed for about 24 h (Figs. 2a, 2b) which can be attributed to adjustment of cells to new environmental conditions during storage (4 ºC) compared to their prior culturing and growth temperatures of 37 and 21ºC, respectively. Further decreasing the culture growth temperature to 4 ºC which we regarded as a bit cold-stressed condition and subsequent inoculation on different produce showed almost a similar trend as cells that were pre-cultured at 21 ºC except in case of mixed salad (1.25 log reduction) and

carrot (0.45 log increase) (Figure 2c). These findings indicate that pre-growth environmental conditions affect growth, survival, and/or death kinetics of *L. monocytogenes* on fresh-cut salad during refrigerated storage. *L. monocytogenes* can be able to better survive if the pre-growth culture temperatures are 4 or 21 ºC compared to commonly used culture growth temperatures of 37 ºC.

Effect of pre-growth temperature and type of commodity on survival kinetics of *S. enterica*

To further understand the effect of the type of pathogen and their dependence on culture pre-growth temperature, a follow-up study was conducted on *S. enterica*. Fig. 3 shows the survival kinetics of *S. enterica* that were pre-cultured at 37 (Figure 3a), 21 (Fig. 3b), and 4 (Figure 3c) ºC, respectively and subsequently inoculated on different types of produce during simulated retail storage at 4ºC and 80% RH for up to 72 h. Unlike, *L. monocytogenes*, pre-growth temperatures and the type of produce did not show a significant ($P>0.05$) effect on the survival kinetics of *Salmonella*. Within the same produce type, *Salmonella* levels neither increased nor decreased during the storage time (Fig 3a-c). In general, red cabbage showed the least survival rate when compared to other tested produce. Vandamm, Li, Harris, Schaffner, & Danyluk (2013) reported the -0.15 log CFU/g growth of *Salmonella* on cut celery stored in containers at 4°C for 7 days. The findings of our study indicate *S.* enterica was unaffected due to pre-growth temperatures within the tested storage period.

APC, yeast, and mold counts

Table. 2 shows the APC, yeast, and mold counts on the iceberg lettuce, red cabbage, grated carrot, and mixed salad samples during refrigerated storage for 72 h. No significant ($P>0.05$) change in the APC was observed during storage within and across different produce tested. The red cabbage was found to have lower APC though not statistically different from

other produce at the end of 72 h. Nagarajan et al (2017) reported low APC levels in red cabbage and it was attributed to the activity of antimicrobial polyphenolic oxidase. Under similar conditions in the current study, yeast showed a higher count than APC and mold. Yeast levels, non-significantly, increased to their highest count till 48h of the storage time in all tested produce except in iceberg lettuce. A similar higher and significant increment in mold count was observed in shredded carrot (1.13 log CFU/g) and mixed salad (1.04 log CFU/g) by the end of the storage period except with declined levels in iceberg lettuce and red cabbage. Yeast showed a higher overall magnitude over storage than APC and mold levels. Similar levels of yeast and mold count (4.1 log and 3.85-6.7 CFU/g) were recorded on spinach leaves stored at 5 °C (Najafi, H., Mohammad, B., & Bahreini 2012, Li et al., 2017). As per our observation, the tested produce maintained its freshness during the storage period and no visual deterioration or quality changes were observed. As such the effect of naturally present microbiota on the survival kinetics of pathogens in the tested produce is beyond the scope of the current study, it is a key factor that should not be overlooked. We tried to deduce the potential relation between pathogenic (*L. monocytogenes*, *S. enterica*) and spoilage organisms (APC, Y&M), but the data was just not sufficient to provide any conclusive details.

Modeling survival kinetics of *S. enterica* and *L. monocytogenes* in the tested produce

Table. 3 shows parameter estimates of *L. monocytogenes* and *S. enterica* when pre-cultured at different temperatures and subsequently inoculated onto fresh-cut produce including mixed fresh-cut salad during refrigerated storage. The experimental data were tested to check the goodness of fit using ComBase predictive models using DMFit online tool. Both Baranyi and Roberts and Linear models fitted the data. However, the Baranyi and Roberts model (with no lag) in most cases better fitted the data (R^2 ranging from 0.054 to 0.967 for *L. monocytogenes* and

0.194 to 0.994 for *S. enterica*) with lower standard error of fit when compared to linear models (data not shown). Under the tested conditions, maximum inactivation rates ranged from -0.0296 ± 0.00845 (for *Lm* grown at 37 ºC on mixed salad) to -0.00772 ± 0.0106 (for *Lm* grown at 21 ºC on iceberg lettuce). In the case of *Salmonella*, maximum inactivation rates ranged from -0.0328±0.00718 (for cells grown at 4 ºC on carrot) to -0.00796±0.00791 (for cells grown at 37 ºC on red cabbage).

Conclusions

This study investigated the effect of culture pre-growth temperatures (37, 21, and 4 ºC) on the subsequent growth and/or survival kinetics of *L. monocytogenes* and *S. enterica* in fresh-cut produce during short-term simulated retail or consumer refrigerated storage conditions. Under the tested conditions, no significant growth or death of tested pathogens was observed among the tested produce. However, both pathogens survived efficiently in the tested produce irrespective of their inherent chemical profiles. However, the rate of survival was found to be dependent upon the type of produce and the pathogen. Mixed salad less supported the growth of *L. monocytogenes* but showed no effect on *S. enterica*. Culture pre-growth temperatures showed some effect in the case of *L. monocytogenes* but not on *S. enterica.* The findings of this study shed some light on the importance of considering appropriate pre-growth environmental conditions to better understand the growth and survival kinetics of foodborne pathogens in fresh-cut produce. This type of data will be useful to develop efficient interventions to mitigate the risk. Further studies should be focused on interactions of pathogens with phytochemicals and naturally present microbiota on fresh produce during long-term storage while maintaining the eating quality.

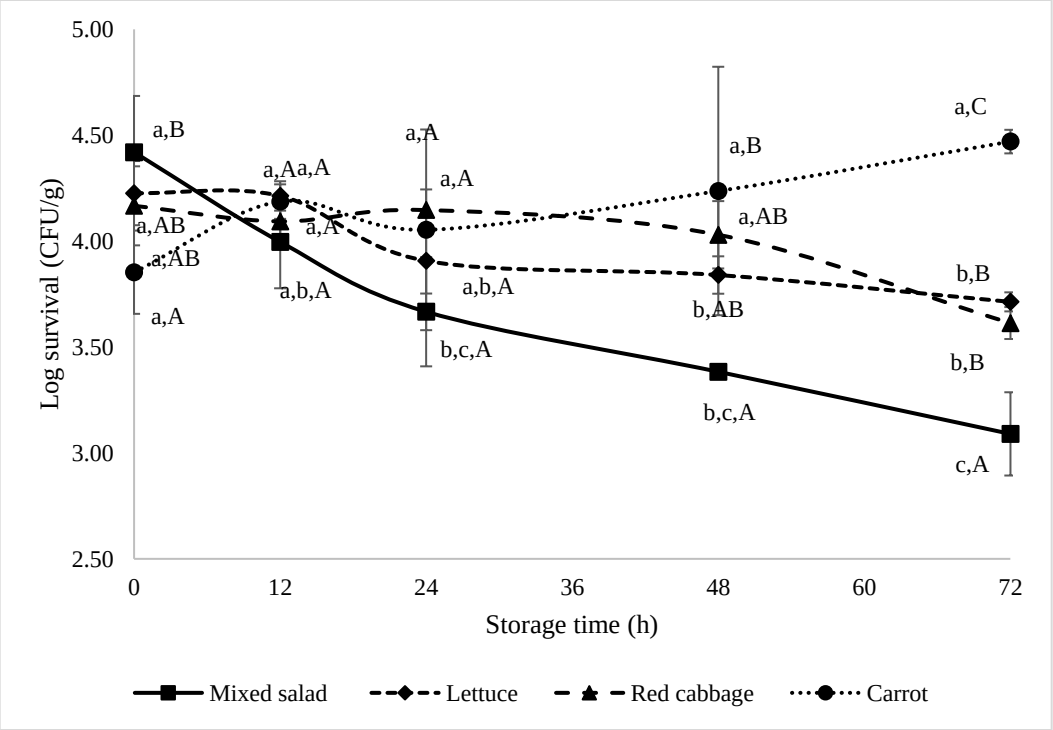

Figure 2a. Survival kinetics of *L. monocytogenes* on iceberg lettuce (♦), red cabbage (▲), shredded carrot (•), and mixed salad (■) when pre-cultured at 37 ºC.

Lower case letters indicate significant differences within the same produce during the storage period, whereas upper case letters indicate significant differences across different produce at a single time point.

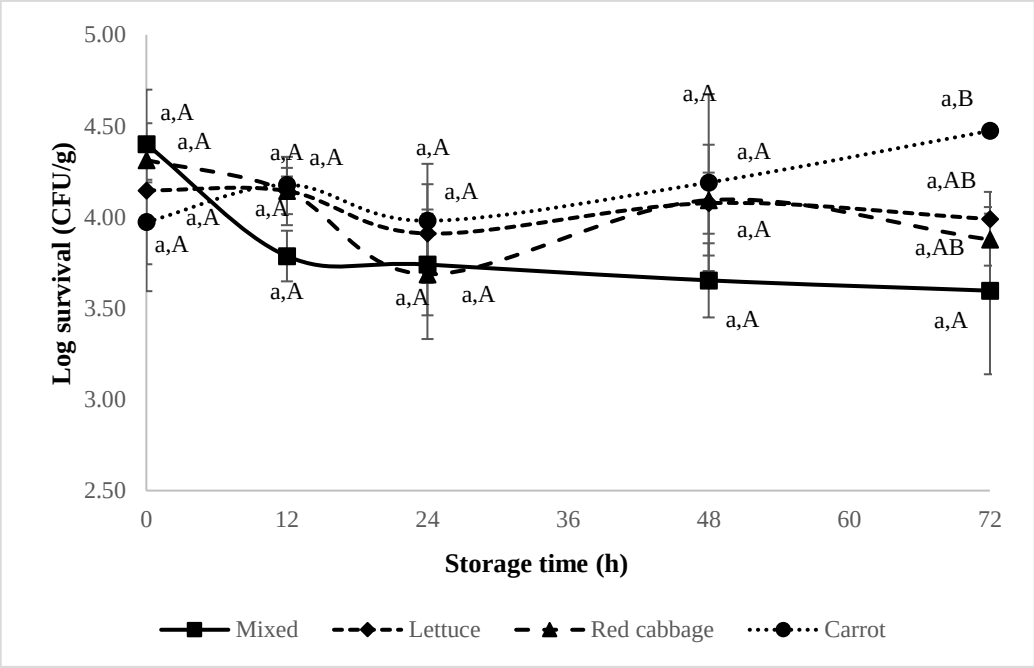

Figure 2b. Survival kinetics of *L. monocytogenes* on iceberg lettuce (♦), red cabbage (▲), shredded carrot (•), and mixed salad (■) when pre-cultured at 21 ºC.

Lower case letters indicate significant differences within the same produce during the storage period, whereas upper case letters indicate significant differences across different produce at a single time point.

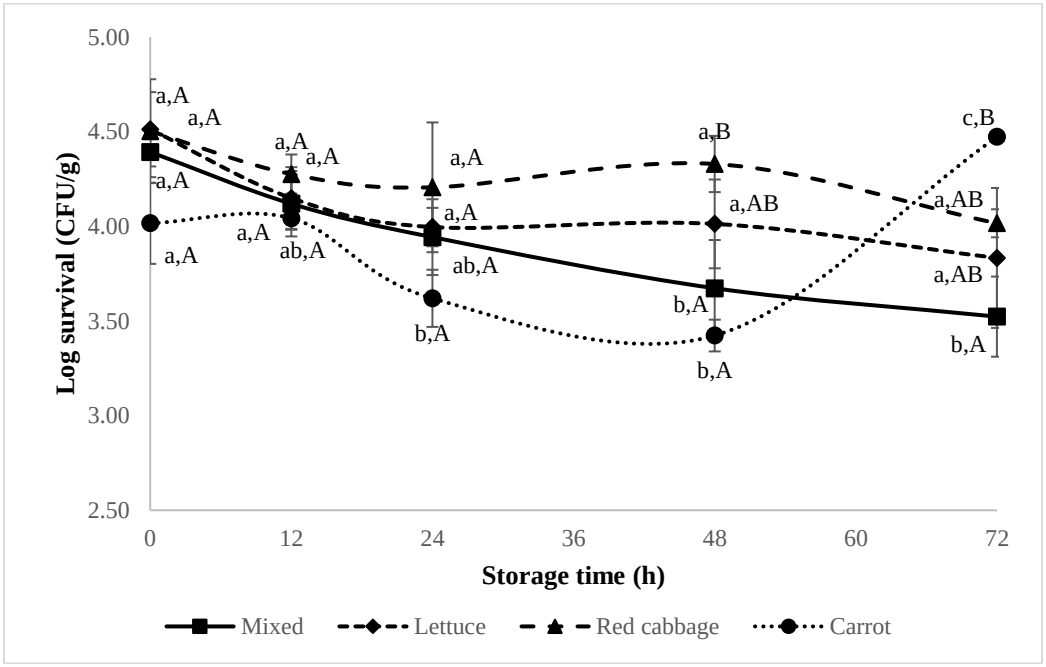

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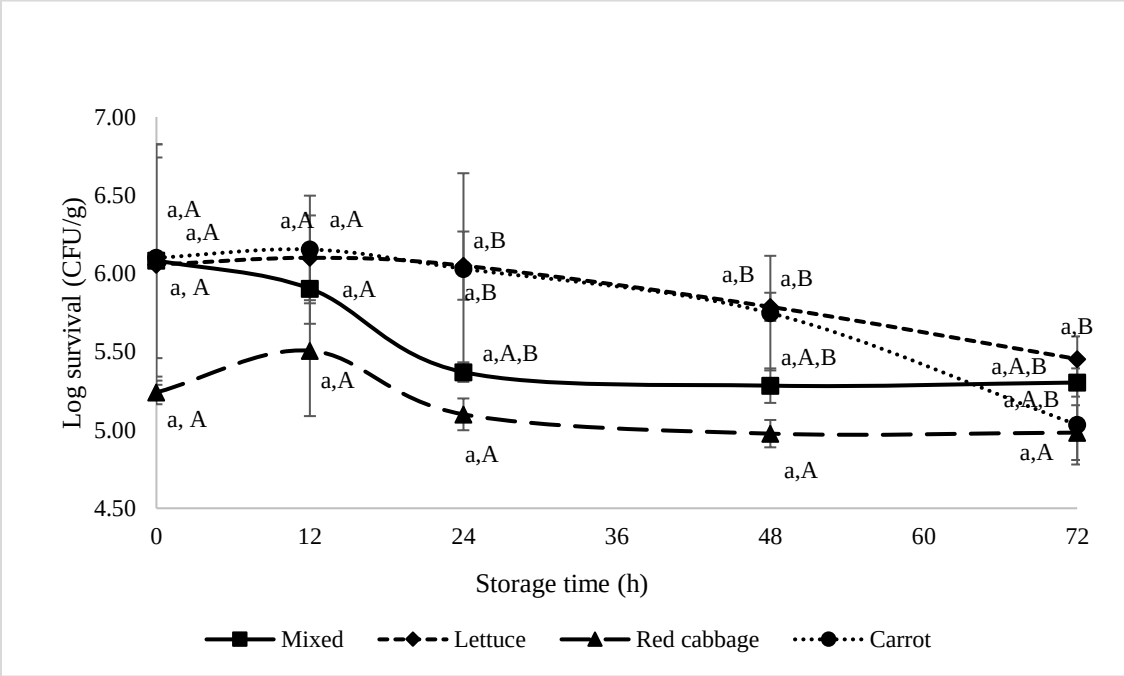
Figure 2c. Survival kinetics of *L. monocytogenes* on iceberg lettuce (♦), red cabbage (▲), shredded carrot (•), and mixed salad (■) when pre-cultured at 4 ºC.

Lower case letters indicate significant differences within the same produce during the storage period, whereas upper case letters indicate significant differences across different produce at a single time point.



42

Figure 3a. Survival kinetics of *S. enterica* on iceberg lettuce (♦), red cabbage (▲), shredded carrot (•), and mixed salad (■) when pre-cultured at 37 ºC.

Lower case letters indicate significant differences within the same produce during the storage period, whereas upper case letters indicate significant differences across different produce at a single time point.

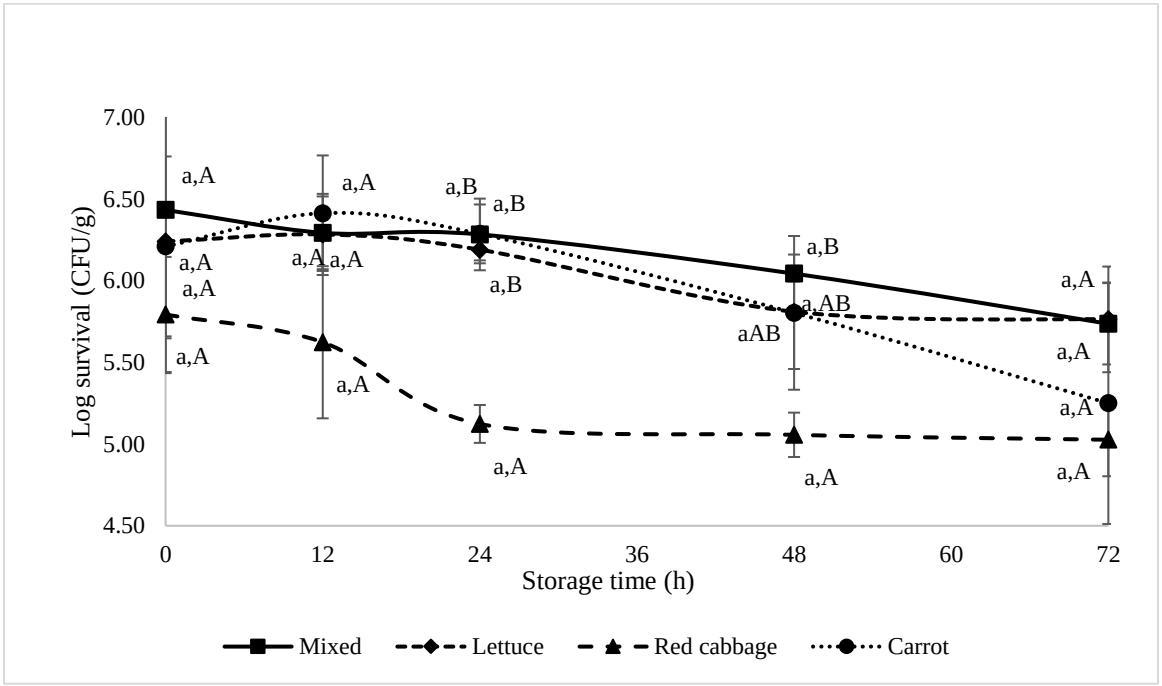


Figure 3b. Survival kinetics of *S. enterica* on iceberg lettuce (♦), red cabbage (▲), shredded carrot (•), and mixed salad (■) when pre-cultured at 21 ºC.

Lower case letters indicate significant differences within the same produce during the storage period, whereas upper case letters indicate significant differences across different produce at a single time point.

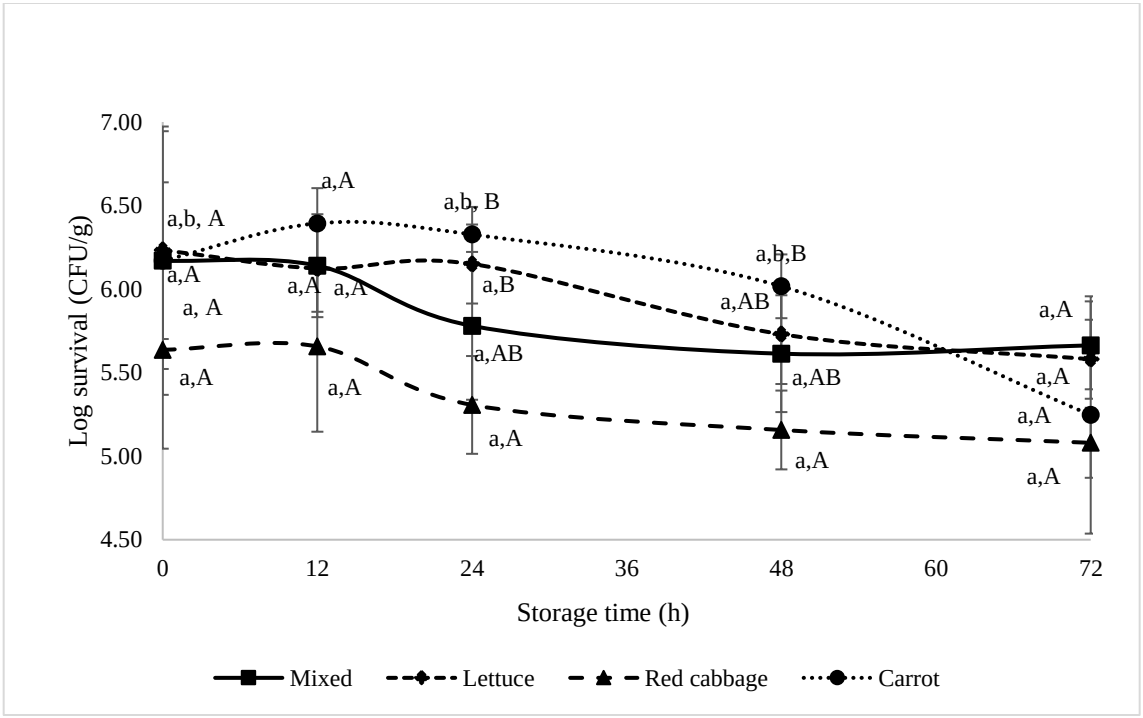


Figure 3c. Survival kinetics of *S. enterica* on iceberg lettuce (♦), red cabbage (▲), shredded carrot (•), and mixed salad (■) when pre-cultured at 4 ºC.

Lower case letters indicate significant differences within the same produce during the storage period, whereas upper case letters indicate significant differences across different produce at a single time point.

Table 2: Aerobic plate counts (APC), Yeast, and Mold counts (log CFU/gm) in control samples during the storage.

Type of organism(s)	Time (h)	Iceberg lettuce	Red cabbage	Grated carrot	Mixed salad
APC	0	$1.88 \pm 1.26^{a,A}$	$2.52 \pm 0.56^{a,A}$	$2.55 \pm 0.25^{a,A}$	$2.60 \pm 0.50^{a,A}$
	12	$2.25 \pm 0.57^{a,AB}$	$1.50 \pm 0.91^{a,B}$	$2.46 \pm 0.29^{a,AB}$	$2.72 \pm 0.27^{a,A}$
	24	$1.32 \pm 0.83^{a,A}$	$1.55 \pm 1.00^{a,A}$	$2.54 \pm 0.32^{a,A}$	$2.14 \pm 0.64^{a,A}$
	48	$2.02 \pm 1.18^{a,A}$	$1.65 \pm 1.23^{a,A}$	$2.47 \pm 0.18^{a,A}$	$2.24 \pm 0.68^{a,A}$
	72	$1.53 \pm 1.01^{a,A}$	$1.08 \pm 0.86^{a,A}$	$2.30 \pm 0.47^{a,A}$	$2.56 \pm 0.40^{a,A}$
Yeast	0	$4.09 \pm 0.15^{a,A}$	$3.99 \pm 0.29^{a,A}$	$4.02 \pm 0.17^{a,A}$	$4.08 \pm 0.06^{a,A}$
	12	$4.09 \pm 0.09^{a,A}$	$4.14 \pm 0.07^{a,A}$	$4.10 \pm 0.05^{a,A}$	$4.10 \pm 0.03^{a,A}$
	24	$4.15 \pm 0.14^{a,A}$	$4.15 \pm 0.12^{a,A}$	$4.16 \pm 0.08^{a,A}$	$4.12 \pm 0.04^{a,A}$
	48	$4.16 \pm 0.05^{a,A}$	$4.23 \pm 0.05^{a,A}$	$4.13 \pm 0.12^{a,A}$	$4.17 \pm 0.16^{a,A}$
	72	$4.06 \pm 0.17^{a,A}$	$4.02 \pm 0.24^{a,A}$	$4.08 \pm 0.23^{a,A}$	$4.14 \pm 0.15^{a,A}$
Mold	0	$0.66 \pm 0.62^{a,A}$	$0.96 \pm 0.57^{a,A}$	$0.94 \pm 0.92^{a,A}$	$0.90 \pm 0.83^{a,A}$
	12	$1.40 \pm 0.55^{a,A}$	$0.52 \pm 0.74^{a,A}$	$2.06 \pm 0.44^{ab,B}$	$1.52 \pm 0.40^{a,A}$
	24	$0.56 \pm 0.76^{a,A}$	$0.46 \pm 0.64^{a,A}$	$2.26 \pm 0.23^{ab,B}$	$1.46 \pm 0.53^{a,A}$
	48	$0.56 \pm 0.76^{a,A}$	$0.82 \pm 0.75^{a,A}$	$1.51 \pm 1.38^{ab,B}$	$1.49 \pm 0.36^{a,AB}$
	72	$0.40 \pm 0.55^{a,A}$	$0.72 \pm 0.67^{a,A}$	$2.07 \pm 1.16^{b,B}$	$1.94 \pm 0.23^{a,A}$

Mean ± standard deviation represented in table (five replications). Lower case letters within a row indicate significant differences within a produce item during the storage period and upper-case letters represent the significant difference among different commodities at a single time point within a row.

Table 3. Parameter estimates of *L. monocytogenes* and *S. enterica* survival kinetics in different produce using the Baryani-Roberts model.

Type of pathogen	Pre-growth temp[a] (ºC)	Type of Produce	R^2	SE of Fit[b]	Initial value (log CFU/g)	Maximum rate[c] (1/h)
L. monocytogenes	37	Lettuce	0.817	0.0988	4.241 ± 0.0789	-0.00944 ± 0.0033
		Cabbage	0.967	0.0417	4.137 ± 0.0241	-0.018 ± 0.00275
		Carrot*	-	-	-	-
		Mixed salad	0.925	0.143	4.392 ± 0.13	-0.0296 ± 0.00845
	21	Lettuce	0.054	0.0998	4.174 ± 0.0982	-0.00772 ± 0.0106
		Cabbage	0.243	0.209	4.332 ± 0.209	-0.0213 ± 0.0243
		Carrot	0.657	0.119	4.0434 ± 0.0707	0.0122 ± 0.00734
		Mixed salad	0.952	0.0709	4.4 ± 0.0709	-0.052 ± 0.00956
	4	Lettuce	0.847	0.1	4.507±0.1	-0.0291 ± 0.0119
		Cabbage	0.204	0.156	4.5± 0.156	-0.0184 ± 0.0191
		Carrot	0.653	0.18	4.0927 ± 0.165	-0.0166 ± 0.0108
		Mixed salad	0.847	0.1	4.507±0.1	-0.0291 ± 0.0119
S. enterica	37	Lettuce	0.994	0.0215	6.0802 ± 0.0147	-0.0143 ± 0.00111
		Cabbage	0.194	0.199	5.372 ± 0.165	-0.00796 ± 0.00791
		Carrot	0.985	0.0575	6.0964 ± 0.0339	-0.03 ± 0.00349
		Mixed salad	0.920	0.106	6.136 ± 0.0983	-0.0296 ± 0.00702
	21	Lettuce	0.801	0.111	6.33 ± 0.0879	-0.00954 ± 0.00345
		Cabbage	0.919	0.101	5.843 ± 0.0933	-0.0279 ± 0.00667
		Carrot	0.950	0.106	6.318 ± 0.0723	-0.0245 ± 0.00549
		Mixed salad	0.957	0.0564	6.403 ± 0.0529	-0.0105 ± 0.00168
	4	Lettuce	0.876	0.102	6.269 ± 0.0794	-0.0101 ± 0.00301
		Cabbage	0.832	0.11	5.688 ± 0.0922	-0.0122 ± 0.00466
		Carrot	0.939	0.114	6.297 ± 0.0663	-0.0328 ± 0.00718
		Mixed salad	0.860	0.0993	6.225 ± 0.0909	-0.0163 ± 0.00599

[a] Culture growth temperature prior to inoculation on different types of produce; [b] SE of fit, standard error of fit

[c] Maximum Rate: Rate of inactivation (negative value) and growth (positive value).

*Baryani-Roberts models failed to fit the experimental data under these conditions

CHAPTER IV

EFFECT OF PRE-GROWTH ENVIRONMENTAL CONDITIONS ON THE *L. MONOCYTOGENES* RESPONSE TO VARIOUS LIGHT TREATMENTS

Abstract

The purpose of this study was to determine the effect of different pre-growth environmental conditions on the response of *Listeria monocytogenes* to various antimicrobial light treatments. Three strain cocktail of *L. monocytogenes* pre-adapted to different temperatures (4, 37 ºC) and pH (4.5) stresses were subjected to antimicrobial blue light (aBL; 455 nm), UV-A (380 nm), and UV-C (254 nm) light treatments for 15 to 60 min. The recovery of surviving cells on both selective and non-selective media was determined. The results indicate that the cells grown under cold (4 ºC), and acidic (pH 4.5) conditions showed higher overall reduction under UV-A and UV-C treatments compared to cells grown at normal laboratory growth temperature of 37 ºC.

However, aBL treatments did not result in a significant ($P > 0.05$) reduction. Hence, UV-C treatment resulted in greater reductions within a short period followed by UV-A and aBLtreatments. The findings of this study are helpful in better selecting more resistant physiological conditions of pathogens when validating antimicrobial intervention treatments.

Keywords: *Listeria monocytogenes*, pre-growth environmental conditions, UV irradiation, antimicrobial blue light, inactivation, light treatment

Introduction

L. monocytogenes is one of the most problematic foodborne pathogens of concern. It has been implicated in multiple foodborne illness outbreaks related to fruits, vegetables, dairy, poultry, and seafood in the past years (Pouillot et al., 2016; Gourama, 2020; Evans, Samuel, Redmond, & Taylor 2021). Several conventional methods such as cleaning, sanitation, disinfection, and pasteurization were commonly followed to maintain hygienic conditions and reduce the risk of contamination (Huang, Chang, & Wang 2015; Marszałek, Mitek, & Skąpska, 2015). Buchanan, Gorris, Hayman, Jackson, & Whiting (2017) reported that *L. monocytogenes* exhibit resistance to temperature, and disinfectant treatments by acquiring new genes and mutations in existing genes. Thermal treatments pose a risk of damaging nutritional components, enzymes, texture, and taste of foods (Morales et al., 2015; de Oliveira, Cossu, Tikekar, & Nitin, 2017; Al-Juhaimi et al., 2018; de Jesus, Leite, & Cristianini, 2018). Thus, non-thermal methods are preferred nowadays due to their less and/or non-detrimental effects on food quality.

Ultraviolet (UV) irradiation processing is a novel, non-thermal, and efficient method of decontaminating food products by inactivating foodborne pathogens. UV-C (200-280 nm) penetration cause the formation of pyrimidine dimers in bacterial genetic material, resulting in hindrance in DNA replication and ultimately cell death (Franz, Specht, Cho, Graef, & Stahl, 2009; Gomez-Lopez, Koutchma, & Linden, 2012). While UV-B radiation (280–315 nm) induces protein damage leading to population reduction and UV-A (315–400 nm) causes sublethal injury to the cells, instead of killing the microbes (Jeon & Ha, 2018). Moreover, due to their germicidal abilities, UV-A and UV-B require long exposure from days to weeks (Moreno-Andrés, Tierno-Galán, Romero-Martínez, Acevedo-Merino, & Nebot, 2023) and UV-C require a relatively short period of time (Kim, Yoon, Lee, Kim, & Lee, 2023) to achieve significant reductions. Many studies have reported the efficacy of UV light treatment on the inactivation of foodborne pathogens (Xu, Wang, Xu, Liu, & Li (2016); Kim, Lee, Kim, & Rhee (2018); Park & Ha (2019); Kim & Kang (2020); Kim, Yoon, Lee, Kim, & Lee (2023)). Ortiz-Sola, Valero, Abadias, Nicolau-Lapeña, & Viñas (2022) reported 3 (*L. monocytogenes*) and 3.6 (*S. enterica*) log CFU/g reductions on fresh strawberries upon exposure to UVC (1.3 kJ/m^2) for 2 min. UV-A light has shown a 1.14 log (*E. coli* O157:H7), 1.11 log (*Salmonella* Typhimurium), and 0.60 log (*L. monocytogenes*) reductions in PBS upon exposure for 90 min (Jeon & Ha, 2020). However, the highly potent UV-C radiation imparts carcinogenic effects which could cause mutations to human DNA, and is harmful to the workers (Blum, 2015). Alternatively, visible light offers a much higher penetration depth with no harmful effects on humans (Schmid, Hoenes, Vatter, & Hessling, 2019). Blue light particularly in the 405 to 470 nm wavelength range has been efficacious to have bacterial suppression over the proper intervals (Bumah, Masson-Meyers, Cashin, & Enwemeka, 2015). Luksiene & Buchovec (2019) reported the effectiveness of visible

light (405 nm) in conjugation with Chlorophyllin-Chitosan to reduce the growth of *L. monocytogenes* (7 log in 2 min) and *E. coli* 157:H7 (4.5 log in 1 h) on the chlorophyllin-chitosan coated strawberries. *L. monocytogenes* not survived on BHI agar and broth when exposed to blue light for 24 h at an intensity of 2.5 mW/cm^2 (O'Donoghue et al., 2016).

In most studies, the physical light treatments to inhibit bacterial growth on fresh produce, storage, or packaging industry surfaces and biofilms have been conducted irrespective of taking into consideration of their effect on bacterial cell proliferation even under different physiological unfavorable conditions. Since *L. monocytogenes* can grow under harsh environments, different physiological conditions (other than 37 °C) exert stress on bacterial cells. Most work has been focused on the survival kinetics of foodborne pathogens grown at a normal laboratory temperature of 37 °C, limited data is available on the response of *L. monocytogenes*, pre-grown under low temperature and pH conditions, to the antimicrobial light interventions. Therefore, the objective of our present study is to investigate the effect of pre-growth temperature stress and *in-vitro* antimicrobial light treatment on the viability of *L. monocytogenes.*

Materials and Methods

Bacterial strains

In this study, a three-strain cocktail of *L. monocytogenes* (i. F8027, serotype 4b, celery isolate; ii. 101M, serotype 4b, beef-associated outbreak isolate; and iii. F8385, serotype 1/2b, carrot isolate) were used as test pathogens. The stocks of all the strains were stored at -80 ˚C containing tryptic soy broth (TSB, Hardy diagnostics, CA, USA) and 25% glycerol (wt/wt). The

frozen stocks of selected strains were thawed at room temperature in a biosafety cabinet and activated by transferring a loopful of inoculum into 10 mL tryptic soy broth with 0.1% yeast extract (TSBY) and pH 4.5 adapted TSBY (using lactic acid). The cultures were then grown under different temperature conditions as discussed below.

Pre-growth environmental stress adaptation and inoculum preparation

Cells were first adapted to three different conditions (37˚C, 4˚C, and pH 4.5) by growing to an early stationary phase. For (i) normal (37 ˚C,) and (ii) acid adaptation (pH 4.5), the cells were grown at 37 ˚C for 18 h and 48 h, respectively. Whereas for cold-adaptation, cells were first grown at 37 ºC for 8 h and subsequently incubated at 4 ºC for up to 40 h to achieve desired working cell concentration. The differentially adapted cells were then centrifuged at 4000 X g for 10 min at 4˚C (Centrifuge 5920R, Eppendorf™, Hamburg, Germany) by taking equal volumes (5 mL) from each strain culture to make a three-strain cocktail. The resultant pellets were re-suspended in 10 mL of 0.1% BPW (Buffered Peptone Water, Hardy Diagnostics, CA, USA), and working inoculum concentration (10^6 CFU/mL) was achieved by serial dilutions. Cell concentrations were confirmed by plating 100 µL portions of appropriate serial dilutions on non-selective (tryptic soy agar; TSA) and selective media (Oxford *Listeria* agar) and incubation at 37 ºC for 24 h.

Light treatment and enumeration

To conduct the light treatment, UV-C (254 nm, 1.7 mW/cm^2), UV-A (380 nm, 4 ± 0.5 mW/cm^2), and aBL (455 nm, 79 mW/cm^2) were used in this experiment. An aliquot of 2 mL of stress-adapted cells in BPW was taken into a sterile PVC cup of size and subjected to respective light treatments for selected time periods. Based on the potential expected efficacy of these light

treatments, we choose different treatment times. For example, aBL and UV-A treatments were performed for up to 60 min while UV-C treatment was performed for up to 15 min. Samples were collected at regular intervals and the survivors were enumerated by plating on both non-selective (tryptic soy agar, TSA) and selective (Oxford) media and incubation at 37°C for 24 h. The survivors were expressed as log CFU/mL.

Statistical analysis

All the experiments were conducted in triplicates and duplicate samples were included in each replication. The CFU data were transformed into log CFU/mL and arranged to conduct statistical analysis to compare the log survivals in the same pre-growth conditions over treatment time and log survivals across different pre-growth environmental conditions at a one-time point. Data were analyzed by the analysis of variance (ANOVA) procedure using SPSS™ (Version 28, IBM®). Tukey's multiple comparison test was performed to determine the mean differences. All the tests were performed with a 0.05 level of significance.

Results and Discussion

Effect of pre-grown conditions on the survival of *L. monocytogenes* under UV-C light

Figure 1 a-b shows the efficacy of UV-C treatment on *L. monocytogenes* preadapted to low temperature (4 ºC) or low pH (4.5) and compared with normally grown cells at 37 ºC on both non-selective and selective media, respectively. A maximum reduction of 5.08, 5.07, and 6 log CFU/mL were observed for 37 ºC, 4 ºC, and pH-adapted cells, respectively after 10 min of treatment (Fig 1a). Further increasing treatment time for up to 15 min, no significant ($P>0.05$) increase in the log reductions was observed. As expected, increasing the treatment time from 0 to 10 min increased the log reductions. However, among the tested pre-growth environmental

conditions no significant difference in the reductions was observed at the end of 15 min treatment time. The germicidal effect of UV light is particularly due to its penetration into DNA and subsequent formation of photoproducts including cyclobutane-pyrimidine dimers, and pyrimidine 6-4 pyrimidine photoproducts) (Cadet, Grand, & Douki, 2015). pH-adapted cells were found to be more susceptible when compared with other pre-growth conditions, but the difference is not statistically significant under the tested conditions. McKinney, Williams, Boardman, Eifert, & Sumner (2009) reported that acid-adapted (pH 5) *L. monocytogenes* showed a reduction by 1.62 (in sterilized distilled water) and 1.5 (in brine solution) log CFU/mL after 30 min UV-C exposure. Bucur, Grigore-Gurgu, Crauwels, Riedel, & Nicolau (2018) reported that *L. monocytogenes* could proliferate under acidic conditions potentially due to the activity of glutamic acid decarboxylase, F0F1-ATPase, and arginine. While the same treatment samples when enumerated on selective media no survivors were detected (>6-7 log reduction) at the end of the treatment period (Fig 1b). It should be noted that recovery on selective and non-selective media showed differences. This can be attributed to the ability of partially injured cells to recover on the non-selective media while the selective Oxford media might not be provided with that opportunity due to the presence of growth inhibitors. A study by Xuan et al (2017) and Garcia et al (2022) reported an approximately 1-log difference in the recovery of *L. monocytogenes* and *Salmonella* between the selective media and the non-selective media after treatment with acidic electrolyzed water and antimicrobial gasses, respectively. Since non-selective media allows resuscitation of all injured cells whereas selective media inhibits the growth of injured cells due to the presence of selective agents.

Effect of pre-grown conditions on the survival of *L. monocytogenes* under UV-A light

Figure 2 a-b shows the efficacy of UV-A (380 nm) light treatment on *L. monocytogenes* under the previously mentioned pre-growth environmental conditions. Under tested conditions, a maximum reduction of 0.88 (37 ºC, 30 min), 0.92 (low pH adopted; 45 min), and 0.52 (4 ºC, 45 min) log CFU/mL was observed. Further increasing the treatment time for up to 60 min, no significant (*P>0.05*) difference in log reductions was observed. Similar results of *L. monocytogenes* population reductions in PBS followed by 90 min UV-A irradiation were observed (Jeong & Ha, 2019; Jeon & Ha, 2020). UV-A is known to produce ROS (Agüero, Jagus, Martín-Belloso, & Soliva-Fortuny, 2016) and induces sublethal effects without killing the bacteria by protein damage, and reduced energy metabolism (Bosshard et al., 2010). The lower extent of bacterial population reduction by UV-A light could be attributed to the thicker peptidoglycan layer of gram-positive *Listeria*, acting as a permeability and oxidative damage barrier (Park & Kang, 2021). When the same samples were enumerated on selective media, low temperature adapted cells (4 ºC) showed the highest reduction of 1.24 log CFU/mL after 60 min treatment time followed by low pH adapted cells (0.74 log CFU/mL).

Effect of pre-grown conditions on the survival of *L. monocytogenes* under aBL light

Figure 3 a-b depicts the efficacy of aBL (455 nm) light treatment on *L. monocytogenes* under the previously mentioned pre-growth environmental conditions. Upon aBL treatment, no pronounced effect of different pre-growth conditions was shown on the survival of *L. monocytogenes*. The 37 ˚C adapted cells of *L. monocytogenes* showed the highest reduction (0.72 log CFU/mL) on non-selective media after exposure to aBL light for 60 min. The bacterial cells adapted to low temperature and acidic conditions, on the contrary, showed increased levels of injured cells after 60 min of light treatment. The findings suggested that exposure time should

exceed 60 min for the effective mitigation of bacterial cells using aBL light. Ramakrishnan, Maclean, MacGregor, Anderson, & Grant (2016) explained that visible light (400–470 nm) treatment induces photoexcitation of porphyrins and flavin leading to the ROS generation in bacteria, ultimately damaging both cellular proteins and membrane. McKenzie, Maclean, Timoshkin, MacGregor, & Anderson (2014) reported a 5-log reduction in acid-stressed (pH 3) *L. monocytogenes* upon 405 nm light irradiation and concluded that cold-stressed cells (4 °C) showed higher bacterial inactivation than the normally grown bacteria (37 °C). Kang et al (2019) reported that illumination of 405 nm LED to *L. monocytogenes* for 150 min, disrupted the bacterial cell membrane and declined its count from 4.6 to 4.2 log CFU/mL after its exposure for 96 h under 4°C. Enumeration of the same light-treated cells on selective media showed the maximum reduction of low-temperature adapted cells by 0.56 log CFU/mL after 30 min of treatment. The bacterial count of acid-adapted, and normally grown *L. monocytogenes* did not show distinct reduction throughout the treatment time up to 60 min. Kim, Da Jeong, Zheng, & Yuk (2021) studied the illumination of 405 nm LED light (57.6 J/cm^2) on the PBS containing *L. monocytogenes* where the population was reduced by 1.9 log CFU/mL. Lee, Kim, & Kang (2023) reported the inactivation of *L. monocytogenes* upon exposure to blue light (80 J/cm2) from 7.20 to 4.01 log CFU/mL.

Conclusions

In this present study, we have investigated the response of pre-growth-adapted *L. monocytogenes* to different antimicrobial light treatments. Since *L. monocytogenes* uses diverse mechanisms to survive under various conditions, the results showed that cells grown under various environmental stresses behave differently under light treatments. Our results concluded

that UV-C has a higher potential to inactivate the bacterial cells followed by UV-A. However, antibacterial blue light had the least impact on the inactivation of *L. monocytogenes* cells due to minimum treatment time. Also, among the stresses, the low-temperature adapted *L. monocytogenes* showed higher bacterial reductions than acidic and normally grown cells. Future directions should focus on the *in vivo* treatment of gram-positive and gram-negative bacteria with visible blue light for longer exposure time to differentiate their ability to respond or resist.

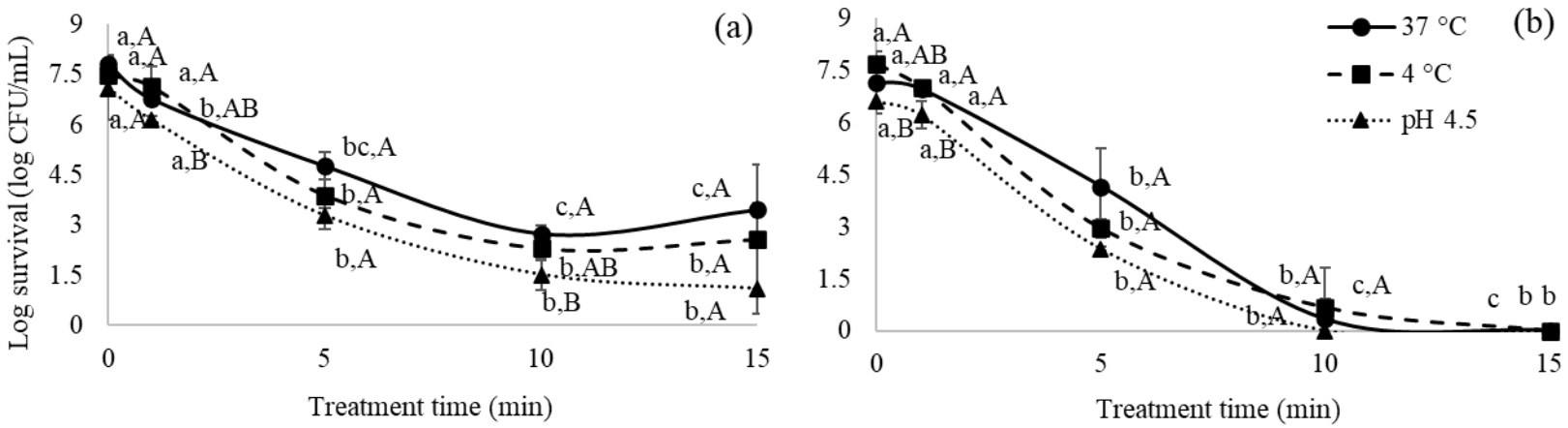


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Figure 4: Efficacy of UV-C light treatment on *L. monocytogenes* pre-grown 37 ºC (•), 4 ºC (■) and pH 4.5 (▲) conditions when recovered on (a) non-selective and (b) selective media, respectively.

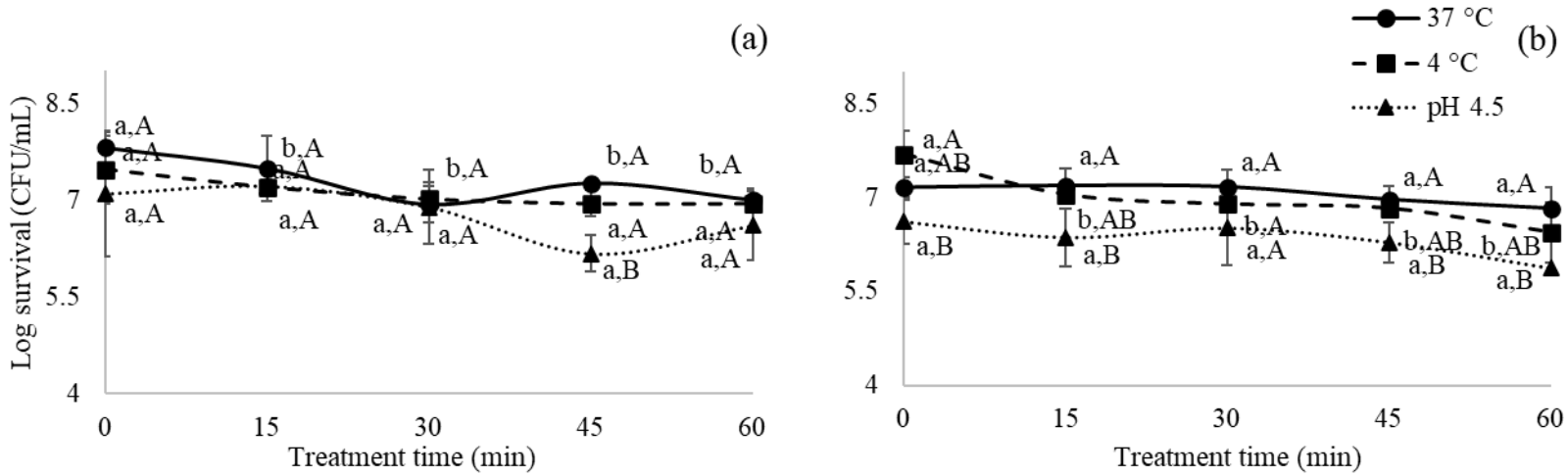

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Figure 5: Efficacy of UV-A light treatment on *L. monocytogenes* pre-grown under 37 ºC (•), 4 ºC (■) and pH 4.5 (▲) conditions when recovered on (a) non-selective and (b) selective media, respectively.

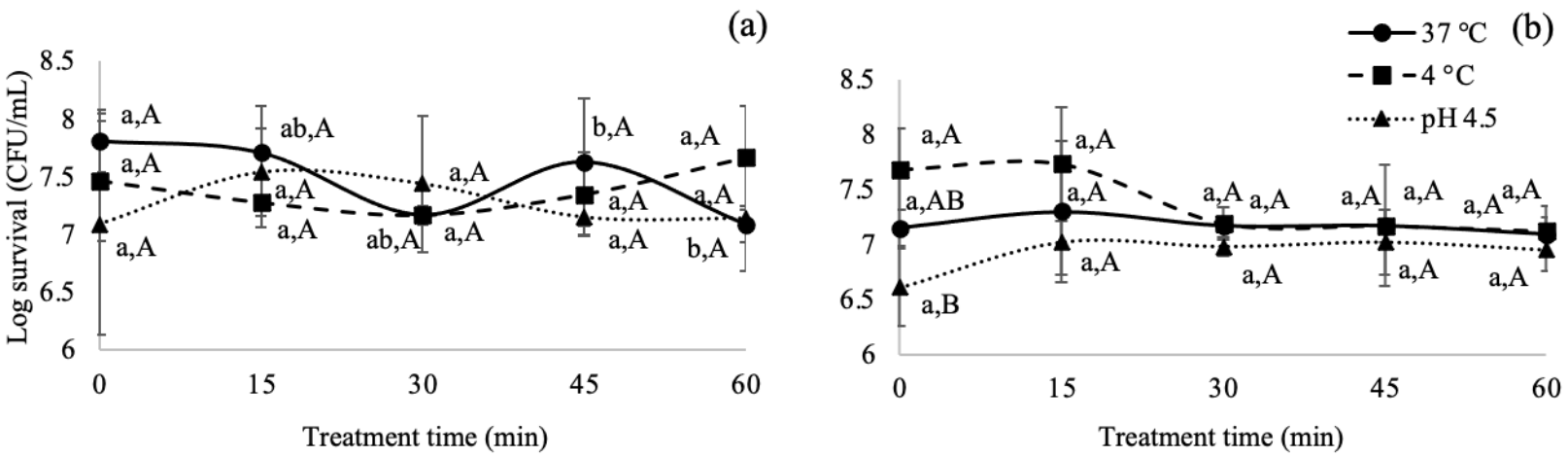

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Figure 6: Efficacy of aBL light treatment on *L. monocytogenes* pre-grown under 37 ºC (•), 4 ºC (■), and pH 4.5 (▲) conditions when recovered on (a) non-selective and (b) selective media, respectively.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

With increasing consumption of minimally processed fresh produce, the number of foodborne outbreaks associated with these has also risen. It has been challenging to study and timely intervene the fresh produce related outbreaks due to their shorter shelf-life, delayed identification of specific outbreak pathogen, and so on. CDC has reported the *L. monocytogenes* and *S. enterica* has been implicated in the foodborne outbreaks linked to pre-packaged salads, leafy greens, lettuce and papaya in past years. A number of studies have been conducted to determine the growth kinetics of these foodborne pathogens grown under normal laboratory temperature condition 37 °C. However, it is not the case in real-life scenario that fresh products are always subjected to ambient conditions. The associated pathogens may experience a change in optimal conditions either during storage or distribution which could impact their survival in fresh produce under diverse conditions. Surprisingly, *L. monocytogenes* and *Salmonella* could proliferate or survive under harsh conditions such as high or low temperature, alkaline or acidic environments due to the expression of specific genes or factors. Limited literature is available on the growth and survival kinetics of pathogens grown at conditions other than 37 °C prior inoculation on the fresh-cut produce.

Therefore, this thesis project was conducted to accomplish two major goals including (1) effect of different pre-growth temperatures on the survival and growth kinetics of *S. enterica* and

L. monocytogenes in fresh-cut salad during refrigerated storage; (2) effect of pre-growth environmental stresses on the *L. monocytogenes* response to various antimicrobial light treatments. The findings of present work revealed that *L. monocytogenes* grown at 37 °C showed reduced levels in iceberg lettuce, red cabbage and mixed salad at the end of 72h storage; and non-significant changes were observed in inoculated grated carrot under storage at 5 °C and 80 ± 2% RH. Th reduction in population could be attributed to (i) the depletion of nutrients by the end of storage period ultimately slowed the growth; and/or (ii) release of phytochemicals such as polyphenols acting as potent antimicrobials against the survival of pathogen. Non-significant fluctuations were observed in the survival kinetics of 21 and 4 °C grown *L. monocytogenes*. Among all the fresh cut commodities, mixed salad showed the highest reduction in bacterial population followed by red cabbage and lettuce, except grated carrots possibly due to cooperative effect of different metabolites present in individual commodities. This study concluded that produce type significantly impacts the survival of foodborne pathogens. *Salmonella* grown at 37, 21, and 4 °C did not show much effect on its growth or survival kinetics on the fresh-cut produce, where carrot showed the highest reduction (37ºC) followed by mixed salad (37ºC), red cabbage (21ºC), and iceberg lettuce (4ºC), respectively. APC, yeast and mold count showed a non-significant change except mold in all tested produce stored under similar conditions. Baranyi-Roberts and Linear mathematical models were tested to fit the experimental data and determine the predictive power of these models for *Salmonella* and *L. monocytogenes* kinetics in fresh-cut salads. Baranyi-Roberts showed a better fit than Linear model for our data. Understanding the survival behavior of foodborne pathogens, next logical step is to develop novel interventions to mitigate their growth where we analyzed the response of pre-growth environmental stresses on the *L. monocytogenes* to antimicrobial light treatments. We used

temperature 37 and 4 °C, and pH 4.5 for the adaptation of bacteria during growth, which then treated with UV-A, UV-C, and aBL. The results revealed that pre-growth environmental conditions showed significant ($P \leq 0.05$) effect on the survival kinetics of *L. monocytogenes* upon treatment with UV lights, whereas less pronounced effects were observed after aBL treatment. The cells grown at low temperature showed higher population reduction than the cells adapted to acidic conditions. The results also concluded that longer exposure time under aBL could have possibly eliminated the microbial population to a higher extent. These findings indicate that appropriate selection of pre-growth environmental conditions is critical to better understand the kinetics of foodborne pathogens, also when validating antimicrobial intervention treatments to reduce the contamination in order to ensure food safety.

The data generated through this thesis work provide vital information to understand pathogen kinetics under various pre-growth conditions and help mitigate the risk of *Salmonella* and *L. monocytogenes* in fresh-cut produce supply chain. However, this does not replace the need for effective pre-harvest, during the harvest and after the harvest controls in the production and processing environments. Future directions should focus on understanding the role of potential natural microbiota in the survival of pathogens in terms of competition or supporting growth. Also, elucidation of biophysical mechanisms of interactions between fresh-cut produce and pathogen will help to better understand their attachment and ultimately, in developing sustainable interventions to lower the pathogenic contamination to fresh produce. These mechanisms would help us to understand the role of different biomolecules in the pathogenicity of selected bacteria along with their interaction to the phytochemicals released by fresh produce.

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BIOGRAPHICAL SKETCH

Avninder Kaur was born and raised in Punjab, India. She completed high school from Govt. Sen. Sec. Model School, Punjab Agricultural University, Ludhiana, Punjab, India in 2015. She obtained her 5-Year Integrated Master's in Biochemistry in 2021, with a major in post-harvest technology for Fruit crops and a research thesis entitled "*Metabolic profiling of pear fruit at different harvesting dates and storage period*", from Punjab Agricultural University, India. During this time, she got interested in Food Safety and Quality. To pursue higher studies, she decided to join Yemmireddy lab in the United States where she conducted research in Food Safety and Microbiology. She earned a Master's in Biochemistry and Molecular Biology from the University of Texas Rio Grande Valley in August 2023. Her hobbies include cooking, traveling, and reading.

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