Understanding *Listeria* biofilms: formation, ecology, and control in the produce industry

by

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Abstract

Listeria monocytogenes, a major foodborne pathogen, has been a growing concern in the fresh produce industry and is increasingly recognized as a vehicle for transmission of foodborne illnesses. Its persistence and contamination potential are strongly associated with its ability to form biofilms. Hard-to-clean sites in food processing environments can provide favorable conditions for biofilm establishment and survival. Exposure of these biofilms to sublethal concentrations of sanitizers can trigger stress response mechanisms, leading to alterations in gene expression that promote adaptive response. Beyond single-species systems, a further challenge is the frequent occurrence of *L. monocytogenes* in multispecies biofilms, particularly with *Pseudomonas* spp., a common spoilage microorganism. These mixed communities exhibit enhanced resistance and tolerance to sanitizing practices, creating greater challenges for effective control.

Therefore, the main objectives of this study were to: 1) Assess the growth of *L. monocytogenes* biofilms on common food processing surfaces used in the fresh produce industry and evaluate the efficacy of commercially available sanitizers alone or simultaneously with UV-C light; 2) Characterize the transcriptional response of *L. monocytogenes* biofilms exposed to sublethal concentrations of commercial sanitizers and identify mechanisms involved in biofilm adaptation; and 3) Investigate the interactions between *Pseudomonas fluorescens* and *L. monocytogenes* in dual-species biofilms and evaluate the efficacy of commercially available sanitizers against these biofilms across different food-contact surfaces.

Multi-strain *L. monocytogenes* biofilms were grown in a Centers for Disease Control and Prevention (CDC) biofilm reactor on stainless steel, nylon, polyvinyl chloride (PVC), high density polyethylene (HDPE), and Teflon® for up to 96 h. Mature biofilms were exposed to 120 ppm peracetic acid (PAA), silver dihydrogen citrate (SDC; 4.85% citric acid and 0.003% silver ions),

4% lactic acid, and UV-C light (254 nm) for 1 or 5 min by spraying. Biofilm architecture was evaluated using Laser Scanning Confocal Microscopy (LSCM) before and after treatments. Uninoculated surface materials showed different topographical characteristics and when mature biofilms were treated for 1 min with different sanitizers, biomass was visibly affected. Antimicrobial treatment and the interaction of treatment and material showed significant effects ($P < 0.05$). All tested liquid sanitizers significantly reduced biofilm cell populations as compared to controls ($P < 0.05$), with PAA and lactic acid demonstrating the highest efficacy across surfaces. However, simultaneous application of UV-C light with sanitizers did not statistically improve treatment efficacy.

Given the observed variation in sanitizer efficacy against *L. monocytogenes* biofilms populations observed, transcriptomic analysis was conducted to further explore the relationship between sanitizer-induced stress and gene expression. Biofilms were exposed to sublethal concentrations of PAA (10 ppm), chlorine (10 ppm), SDC (4.85% citric acid and 0.003% silver ions), and lactic acid (0.4%), with untreated biofilms serving as controls. *L. monocytogenes* biofilms responded differently to sublethal stresses of these sanitizers. Among them, chlorine and peracetic acid induced the most rapid and significant transcriptional changes. Comparative analysis of differentially expressed genes (DEGs) revealed both unique and shared transcriptional responses across treatments. Chlorine induced the highest number of unique upregulated genes, while chlorine and PAA shared a subset of commonly regulated genes. This suggested both distinct and overlapping stress response mechanisms depending on the sanitizer used. Chlorine exposure triggered regulatory and transport systems, while PAA induced ion transport and ABC transporters. These sanitizer-specific responses suggested distinct adaptive strategies that may contribute to persistence.

Since complex interspecies interactions can enhance resistance and tolerance to sanitation efforts, this study also evaluated the efficacy of commercial sanitizers against dual-species biofilms of *L. monocytogenes* and *P. fluorescens*. Biofilm production capacities of these species were first assessed by crystal violet staining. Dual-species biofilms were then grown in a CDC biofilm reactor on stainless steel, PVC, Teflon, and HDPE surfaces at 22 ± 2°C for 96 h. Mature biofilms were treated by immersion with the previously tested sanitizers, along with chlorine (500 ppm) for 5 min. Surviving cells were recovered on selective and general media. In addition, growth dynamics of both species were monitored over time, and LSCM was used to visualize colonization patterns and spatial organization within the biofilms. Results revealed that both *P. fluorescens* and *L. monocytogenes* were found to be biofilm formers with different production capacities and *P. fluorescens* showed significantly higher biomass production than *L. monocytogenes* ($P < 0.05$). Material, treatment, and their interaction had significant effects on microbial recovery ($P < 0.05$). Among sanitizers tested, chlorine was the most effective showing enhanced effectiveness on stainless steel where the total counts were reduced from 8.43 ± 0.32 log CFU/coupon to 5.26 ± 0.09 log CFU/coupon. A similar trend was observed when *L. monocytogenes* and *P. fluorescens* populations were enumerated individually. PAA was also effective against dual-species biofilms. On HDPE, PAA treatment resulted in the combined counts of 6.27 ± 0.15 log CFU/coupon where untreated control was 8.63 ± 0.34 log CFU/coupon.

Overall, this study demonstrates that *L. monocytogenes* biofilms exhibit sanitizer and surface dependent responses and provides insight into how interspecies interactions and sanitizer adaptations affect their persistence on food processing surfaces. These findings provide insightful information on the use of a combined sanitizing approaches and support the development of more

effective strategies for controlling biofilms on food processing surfaces in the fresh produce industry.

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Dedication

I dedicate this dissertation to Utku and my family.

Chapter 1 - Literature Review

1.1 Physiological Characteristics of *Listeria monocytogenes*

Listeria monocytogenes is a Gram-positive, rod-shaped, and facultatively anaerobic bacterium. Its psychotropic nature allows this microorganism to survive at refrigeration temperatures (-1.5 to 4 °C) (Alejandro-Navarreto & Freitag, 2024), at a broad pH range (4.0 – 9.6) and relatively low water activity ($a_w < 0.92$), making it a significant challenge for the food industry (Osek et al., 2022). *L. monocytogenes* can be found in diverse environments, including soil, decaying vegetation, animal feces, and stream water (Ferreira et al., 2014). This bacterium is able to survive under high salt concentrations up to 11.5% (Nowak et al., 2021) and exhibits motility at temperatures below 30°C but not at 37°C, indicating its temperature-dependent flagellar activity (Abdulkadieva et al., 2023; Gründling et al., 2004).

L. monocytogenes is a genetically diverse species classified into four phylogenetic lineages, with the majority of isolates belonging to Lineages I and II (Finn et al., 2023; Wagner et al., 2022). Within *L. monocytogenes*, 14 serovars have been identified, with at least 95% of strains isolated from food and clinical cases belonging to serovars 1/2a, 1/2b, 1/2c, and 4b (Borucki & Call, 2003; Doumith et al., 2004; Muchaamba et al., 2022). Serotype 1/2a is the most frequent serotype isolated from food, whereas serotype 4b is most commonly linked to human listeriosis cases (Borucki & Call, 2003). Multilocus sequence typing (MLST) has further revealed population structure and distribution of *L. monocytogenes* in clinical and food isolates by classifying them into clonal complexes (CCs) (Muchaamba et al., 2022). Clonal complex 1 (CC1), which belongs to Lineage I, evolved from a subgroup of serotype 4b. This clone has been identified in multiple countries and is frequently associated with various food matrices, including dairy products (Moura, 2021). Bovines, as asymptomatic carriers of *L. monocytogenes*, may contribute to the prevalence of CC1

and potentially facilitates its transmission to fresh produce through soil amendments. (Moura, 2021). CC2, CC4, and CC6 (all from lineage I) of serotype 4b have been also frequently associated with human listeriosis and considered hypervirulent clones (Amarasekara et al., 2024). Strains of *L. monocytogenes* strains from CC1, CC2, CC4, and CC6 (lineage I) possess a genetic advantage, as they encode accessory virulence factors in addition to core virulence determinants (Møretrø et al., 2024). C9 and CC121 isolates belong to lineage II, and they have been recovered from food and food processing environments (Fagerlund et al., 2022; Song et al., 2022; Wagner et al., 2022).

1.2 *Listeria monocytogenes* in the Food Industry

The Centers for Disease Control and Prevention (CDC) estimates that 1,600 people are infected with *Listeria* each year in the United States and 260 people die due to infection (CDC, 2024). Over the past decade, around 30 outbreaks have been reported, leading to 524 cases and 80 deaths (Mohapatra et al., 2024).

L. monocytogenes has been isolated from a wide variety of raw and processed food products, including dairy and meat products, seafood, vegetables, and other ready-to-eat (RTE) products (Ferreira et al., 2014). In RTE products, contamination is often results from recontamination after processing, which of particular concern as these foods typically do not undergo further antimicrobial treatments or kill steps to reduce microbial load (Ferreira et al., 2014; Forauer et al., 2021). The extended shelf life of RTE products can further enable *L. monocytogenes* to grow at hazardous levels during storage (Calvo-Arrieta et al., 2021). Prepackaged contaminated products may also serve as vehicles for *L. monocytogenes* transmission in retail settings, especially during handling (Pradhan et al., 2009). Studies have detected *L. monocytogenes* in various sources at processing and retail level such as ingredients, equipment,

and employees, linking the majority of listeriosis outbreaks to inadequate hygiene practices and unsanitary conditions (Calvo-Arrieta et al., 2021).

Fresh produce is increasingly recognized as a source of listeriosis outbreaks. The prevalence of *L. monocytogenes* on produce is influenced by multiple factors, as contamination can arise from both direct or indirect sources, including animals, soil, water, unsanitized equipment, and human handling (Zhu et al., 2017). Outbreaks are often linked to postharvest contamination, as *L. monocytogenes* can persist in packing or processing facilities (Sullivan & Wiedmann, 2020). Sullivan & Wiedmann (2020) reported that outbreaks are typically attributed to postharvest contamination rather than preharvest due to survival of *L. monocytogenes* at the harborage sites.

Table 1.1. Reported listeriosis outbreaks over the past decade in the U.S.

Year	Associated food product	Total Number of cases	Number of Death	Relevant Information about Outbreaks
2014-2015	Caramel Apples	35	7	Environmental testing of food contact surfaces within the facility revealed *L. monocytogenes* isolates were highly related to outbreak strains (Ward et al., 2022).
2015	Soft cheese	30	3	*L. monocytogenes* was identified in environmental samples from the facility, with WGS confirming a genetic link to outbreak strains (CDC, 2015a).
2015	Ice cream	10	3	*L. monocytogenes* was found in environmental and product samples in the production facilities (CDC, 2015b)
2016	Packaged Salads	19	1	*L. monocytogenes* was detected in packaged salad mix during routine sampling (CDC, 2016a) and all salad mixes produced at the facility were recalled.

2016	Frozen vegetables	9	3	Contaminated products included frozen corns and organic petite green peas. Collected environmental samples from the company contained *Listeria*, which were genetically related to ill people's isolates (CDC, 2016b).
2016	Raw milk	2	1	*Listeria* was detected in raw chocolate milk, resulting in one person's death (CDC, 2016c).
2017	Soft raw milk cheese	8	2	The outbreak strain of *Listeria* was identified in samples taken from three wheels of cheese (CDC, 2017).
2016-2020	Enoki mushrooms	36	4	*L. monocytogenes* was identified in enoki mushrooms imported from a manufacturer located in the Republic of Korea (Pereira et al., 2023; CDC, 2020).
2021	Packaged leafy greens	18	3	*Listeria* was detected on a piece of equipment in the manufacturer's facility, matching the strain found in a positive product sample (FDA, 2022).
2021	Hispanic-style fresh and soft cheeses	13	1	In early 2020, *L. grayi* and *L. innocua* were found in the processing areas, indicating *L. monocytogenes* could also survive in the same environment (CDC, 2022).
2022	Deli meats and cheese	16	1	A specific source was not identified, but contamination likely originated from a food item that introduced *L. monocytogenes* into delis across multiple states (CDC, 2025).
2022	Brie and Camembert soft cheese products	6	0	WGS showed that *Listeria* isolates found in the cooling room were closely related genetically to *Listeria* from sick people's samples (FDA, 2024a).

2022	Ice cream	28	1	*L. monocytogenes* was detected in both environmental and finished product samples from the facility (FDA, 2024b).
2023	Peaches, plums, and nectarines	11	1	*L. monocytogenes* was detected in environmental samples from the facility that packed the recalled stone fruits (FDA, 2024c).
2024	Deli meat	61	10	A notable contributing factor to this outbreak was identified as inadequate sanitation practices in the facility. (USDA, 2025).
2025	Frozen supplemental shakes	42	14	Three of the environmental swabs collected from the processing area tested positive for *L. monocytogenes.* WGS analysis determined that *Listeria* detected in these samples was closely related to the clinical of *Listeria* causing illnesses (FDA, 2025).

Table 1.1. summarizes listeriosis outbreaks that have occurred over the past decade in the U.S. One of the deadliest *Listeria* outbreaks was linked to commercially produced, prepackaged caramel apples in 2014-2015, resulting in seven deaths (20%). Officials suggested that the outbreak was due to a critical failure at the facility or the introduction of *L. monocytogenes*. The pathogen was detected on the packing line, and exposed porous surfaces were present (Angelo et al., 2017). Three different produce-related outbreaks also occurred in 2016, involving contaminated packaged salads and frozen vegetables. Notably, the frozen vegetable outbreak was the first known outbreak of its kind in the U.S (Madad et al., 2023). Investigation findings revealed several potential contamination sources that led to outbreak (CDC, 2016b; Madad et al., 2023). This included facility construction that hindered adequate cleaning of floors and walls, and a failure to prevent condensate from contaminating food contact surfaces. Additionally, the lack of

proper cleaning and sanitation of food contact surfaces provided conditions for microbial growth. Although the original source of contamination remained unidentified, these findings highlighted how deficiencies in cleaning and sanitation practices can lead to deadly outbreaks (Madad et al., 2023). A multistate listeriosis outbreak associated with packaged leafy green salad in 2015-2016 further emphasized the risks related to produce. Inspection findings noted several critical issues: failure to collect environmental samples from food contact surfaces, collecting of environmental samples only before production, and failure to conduct follow-up investigations after identifying *Listeria* spp. from environmental samples over a span of 19 months (Self et al., 2019). Another leafy green outbreak in 2021 resulted in two reported deaths (CDC, 2019), underscoring the significant burden of listeriosis linked to leafy greens. Since *Listeria* spp. are commonly found in soil across various agricultural environments, freshly harvested produce, especially crops that are in close contact with the topsoil are at high risk of contamination (John et al., 2020).

Dairy products have also been involved in recalls and outbreaks due to *L. monocytogenes* contamination. The most common cause of cheese-related recalls and outbreaks has been attributed to *L. monocytogenes* in both the US and Canada. This includes raw milk cheese and cheese made from pasteurized milk, often due to postprocess contamination (Sauders & D'Amico, 2016). During an outbreak linked to contaminated soft cheese in 2015, isolates from environmental samples were found to be closely related to the ones from ill people. In 2023, another listeriosis outbreak that resulted in fatalities serves as a notable example of this risk of frozen products in listeriosis outbreaks even if less frequently implicated compared to soft cheeses and milk (Conrad et al., 2023). In 2023, a deadly listeriosis outbreak linked to milkshakes, was traced back to ice cream machines, which were not cleaned correctly (Washington State Department of Health, 2023).

Overall, these outbreaks highlight the ability of *L. monocytogenes* to persist in food processing facilities through environmental niches and harborage sites. In addition to improved equipment design and rigorous sanitation programs, establishing a robust environmental monitoring program is essential to identify potential contamination sources early and prevent product contamination.

1.3 Structure and Development of *Listeria* Biofilms

Biofilms are complex microbial communities attached to abiotic or biotic surfaces, embedded in an extracellular polymeric substance (EPS) (Hernández-Jiménez et al., 2013; Liu et al., 2023a; Lopez et al., 2010). Most bacterial cells are inherently capable of forming biofilms (Muhammad et al., 2020). Within these structures, bacterial cells exhibit distinct properties such as altered physiology and increased resistance and tolerance to antimicrobials as compared to their planktonic counterparts (Colagiorgi et al., 2016; Toyofuku et al., 2016). Biofilms enable long-term persistence in the environment by protecting the bacterial community and providing a stable microenvironment under adverse conditions (Rather et al., 2021). This enhanced adaptation to nutrient limitation, oxidative stress, antimicrobial agents, and pH fluctuations is largely due to the expression of stress-related genes (Petrova & Sauer, 2012).

Listeria biofilms are mainly composed of cells and EPS, which play a crucial role in surface adhesion and structural integrity of the community. The composition of EPS is highly diverse and influenced by different factors such as bacterial species, strain types, stage of biofilm maturation, and environmental conditions (Campoccia et al., 2021). The specific exopolysaccharides and proteins present often differ between Gram-positive and Gram-negative bacteria (Campoccia et al., 2021). EPS is predominantly composed of polysaccharides, proteins, extracellular DNA (eDNA) and lipids (Donlan, 2002). Among these components, polysaccharides are recognized as

essential structural elements, promoting surface attachment, maintaining biofilm architecture, and providing protection against antimicrobial agents (Brauge et al., 2016).

The ability to form biofilms varies among *L. monocytogenes* isolates, although no consistent correlation has been established with specific lineages or serovars (Borucki et al., 2003). Biofilm architecture is also highly dynamic: shape, structural organization and composition can differ depending on environmental conditions. *L. monocytogenes* biofilms with diverse structural features have been also reported, reflecting the influence of multiple factors such as strain, surface type, growth medium, and temperature (Colagiorgi et al., 2016).

Among the polysaccharides identified, teichoic acids were found to be the major component in *L. monocytogenes* biofilms formed by serotype 1/2a and 4b (Brauge et al., 2016). In another study, an insoluble cell-bound poly-β-(1,4)-N-acetylmannosamine decorated with terminal α-1,6-linked galactose (ManNAc-Gal) was detected as a distinctive structural component (Köseoğlu et al., 2015). Proteins represent another major constituent of *Listeria* biofilms (Ripolles-Avila et al., 2018). Within the matrix, extracellular enzymes contribute to the degradation of biopolymers, while structural proteins establish a network between bacterial surface and EPS, thereby reinforcing matrix integrity (Flemming & Wingender, 2010; Mazaheri et al., 2021). Extracellular surface proteins also play a critical role during initial surface attachment phase (Colagiorgi et al., 2016).

Extracellular DNA (eDNA) constitutes an additional key element of *L. monocytogenes* biofilm matrix, contributing to both stability and integrity (Campoccia et al., 2021; Devaraj et al., 2019; Okshevsky et al., 2015; Seviour et al., 2021). Beyond its structural role, eDNA facilitates horizontal gene transfer, serves as a nutrient source, and binds or sequesters cations (Campoccia et al., 2021; Jakubovics et al., 2013). It also has been suggested that eDNA contributes to biofilm

establishment by enhancing the initial attachment of cells (Zetzmann et al., 2015). Harmsen et al. (2010) showed the significance of eDNA in cell attachment by treating biofilms with DNaseI which is an enzyme that degrades DNA. The DNaseI treated biofilms resulted in reduced cell attachment. Alongside eDNA, EPS represent another major component of the biofilm matrix, with each contributing distinct yet complementary roles in biofilm stability and protection. Beyond its role in enveloping and protecting cells, EPS has several other functions such as cells and aggregation (Mazaheri et al., 2021). EPS is also responsible for the cohesion of biofilms and mediation cell to cell connection while providing hydrated environment (Flemming & Wingender, 2010). EPS furthermore increases biofilm tolerance to antimicrobials and immune cells by limiting their penetration due to the dense layer of polymeric matrix. Therefore, antimicrobials can be inactivated or degraded by the enzymes within the biofilm (Karygianni et al., 2020; Singh et al., 2021).

1.3.1 Initial Attachment and Development

The initial step in biofilm formation is surface attachment (A. Zhao et al., 2023). This phase begins with reversible cell attachment, where free floating cells make a physical contact with the surface. At the first stage, cells are loosely attached and can readily detach and go back to their planktonic form (Fu et al., 2021; Petrova & Sauer, 2012). Motility plays a crucial role in this initial attachment process and various structures can aid bacteria through their movement (Floyd et al., 2017). Non-motile bacteria rely primarily on gravitational sedimentation for attachment, while motile bacteria exhibit varied adhesive properties (Zheng et al., 2021). Particularly, flagella can overcome the resistance that prevents cell surface interactions and enable bacteria to rotate through fluids by swimming or swarming motility (Floyd et al., 2017; A. Zhao et al., 2023).

L. monocytogenes initial attachment is strongly influenced by temperature-regulated motility and surface associated proteins. Flagella are encoded by the *fla*A gene and their synthesis is controlled by temperature (Burdová et al., 2024). They are expressed at temperatures below 30 °C, while their expression is downregulated at 37 °C (Colagiorgi et al., 2016; Renier et al., 2011). Flagella contribute to early attachment, enhance surface interactions and improve colonization efficiency (Renier et al., 2011). However, even in the absence of flagella, *L. monocytogenes* retains a residual adhesion capacity, indicating that additional surface factors can mediate attachment under non-motile conditions through alternative mechanisms such as surface proteins (Tresse et al., 2009).

Environmental stimuli also influence bacterial movement thereby biofilm formation by *L. monocytogenes.* Chemotaxis is a common trait in bacteria, and is defined as the ability of planktonic cells to navigate in fluids along gradients of material concentrations, moving toward chemically stimulating substances such as sugars, amino acids, or other nutrient sources (A. Zhao et al., 2023). Chemotactic signaling to environmental cues may contribute to early stages of biofilm establishment by *L. monocytogenes*. In *L. monocytogenes*, chemotaxis is mediated through the two-component system *CheA/Y*, which tightly links environmental sensing to motility-driven surface colonization (Fan et al., 2020). Importantly, *cheY* mutants display reduced motility and impaired biofilm-forming ability, supporting the idea that chemotaxis is not only critical for locating attachment sites but also for orchestrating efficient surface colonization (Fan et al., 2020).

Hydrodynamics also plays a key role in cell attachment. Shear stress and fluid flow not only influence the transport of cells to surfaces but also shape the resulting biofilm architecture. Moderate flow conditions can enhance colonization by promoting motility or enhancing nutrient availability, whereas higher shear forces may result in cell detachment (Donlan, 2002). The

presence and intensity of shear forces ultimately determine the architecture and resilience of resulting biofilms (Chmielewski & Frank, 2003; Faria et al., 2020).

Surface characteristics, including roughness, hydrophobicity, surface charge, and topography, strongly influence the attachment and colonization of *L. monocytogenes* (A. Zhao et al., 2023). Among these, surface hydrophobicity is considered one of the most prominent factors driving both initial attachment and subsequent biofilm maturation (Manville et al., 2023). Several studies have examined the impact of different surface materials on *L. monocytogenes* biofilm formation (Hua et al., 2019; Manville et al., 2023; Prabhukhot et al., 2024). The role of surface topography is also critical in *L. monocytogenes* adherence to abiotic surfaces. Silva et al. (2008) reported that the bacterium adheres more strongly to surfaces with regular topography, suggesting that uniform patterns provide enhances attachment. Complementing this Takahashi et al. (2010) showed that *L. monocytogenes* also adheres effectively to smoother materials such as glass or PVC, where increased hydrophobicity rather than roughness facilitates stronger interactions. Collectively, these findings indicate that adhesion in *L. monocytogenes* is not strictly determined by a single parameter, but rather by the combined interplay of surface properties.

Irreversible attachment plays a critical role in biofilm formation, representing the transition from a planktonic to sessile lifestyle (Mahto et al., 2022). Adhesion to a surface long enough can signal gene expressions that will then pivot the cellular processes toward biofilm formation. In this stage, cells can be removed by shear forces or chemical breaking.

1.3.2 Accumulation and EPS Synthesis

Attachment to surface induces changes in gene expression, leading to biofilm expansion and EPS synthesis. EPS forms the structural matrix that encapsulates and immobilizes cells, supporting the development of microcolonies (Goel et al., 2021; Mahto et al., 2022). The chemical

and physical properties of EPS vary not only between bacterial species but also among different strains within the same species (Donlan, 2002; Floyd et al., 2017). The formation of structured microbial communities is closely related to the production and quantity of EPS. The mode of life in a biofilm is influenced by several factors, including concentration, cohesion, charge, sorption capacity, and specificity of the individual EPS components, along with the three dimensional architecture of the matrix (Flemming & Wingender, 2010).

Within the biofilm, EPS retains quorum sensing (QS) molecules, providing cell to cell communication (Toyofuku et al., 2016). This communication regulates gene expression, cellular functions, nutrient uptake, and genetic material transfer. In general, QS regulates microcolony formation, biofilm maturation and dispersal (Mahto et al., 2022). The QS system varies between Gram-positive and Gram-negative bacteria. In Gram-negative bacteria, the system is mediated by acyl homoserine lactones (AHLs), while Gram-positive bacteria utilize autoinducing peptides (AIPs), both groups may employ the autoinducer-2 (AI-2) system as a universal signaling mechanism (Preda et al., 2019). These signaling molecules accumulate in the EPS, enabling the regulation of adhesion and the formation of biofilm architecture. During the maturation stage, the production of autoinducer molecules facilitates QS, which controls the surrounding cell density and communal behaviors (Goel et al., 2021). In *L. monocytogenes*, QS is mediated by the luxS dependent AI-2 system and the agrBDCA peptide signaling locus. A study comparing wild-type and QS deficient mutants demonstrated that the mutants exhibited impaired growth under multiple stress conditions, including salinity, temperature, and pH (Banerji et al., 2022).

As *Listeria* biofilm develops, stratification occurs, leading to a more intricate structure (Mahto et al., 2022; Stoodley et al., 2002). Nutrient and chemical gradients, including regions with varying oxygen concentrations, emerge within the biofilm as it develops. Radial expansion of the

biofilm from the initial point of attachment serves as a strategy to enhance access to oxygen and nutrients, especially for cells in deeper layers that might face limitations. The formation of water channels within the biofilm further aids in nutrient transport, ensuring the growth of the community (Floyd et al., 2017).

1.3.3 Dispersal

Bacterial dispersion is a natural stage in *Listeria* biofilm lifecycle (Goel et al., 2021). While a mature biofilm can persist for extended periods, its growth becomes limited over time due to the accumulation of waste materials, depletion of nutrients, and fluctuations in oxygen availability (Goel et al., 2021). Bacteria can decide whether to remain within the biofilm, start colonizing other surfaces to initiate a new biofilm formation process, or return to a planktonic state (Parsek & Greenberg, 2005). There are several mechanisms in the biofilm dispersal stage (Floyd et al., 2017). An increase in cell density is an important factor in dissemination from biofilm, with quorum sensing playing a role in regulating this process (Parsek & Greenberg, 2005).

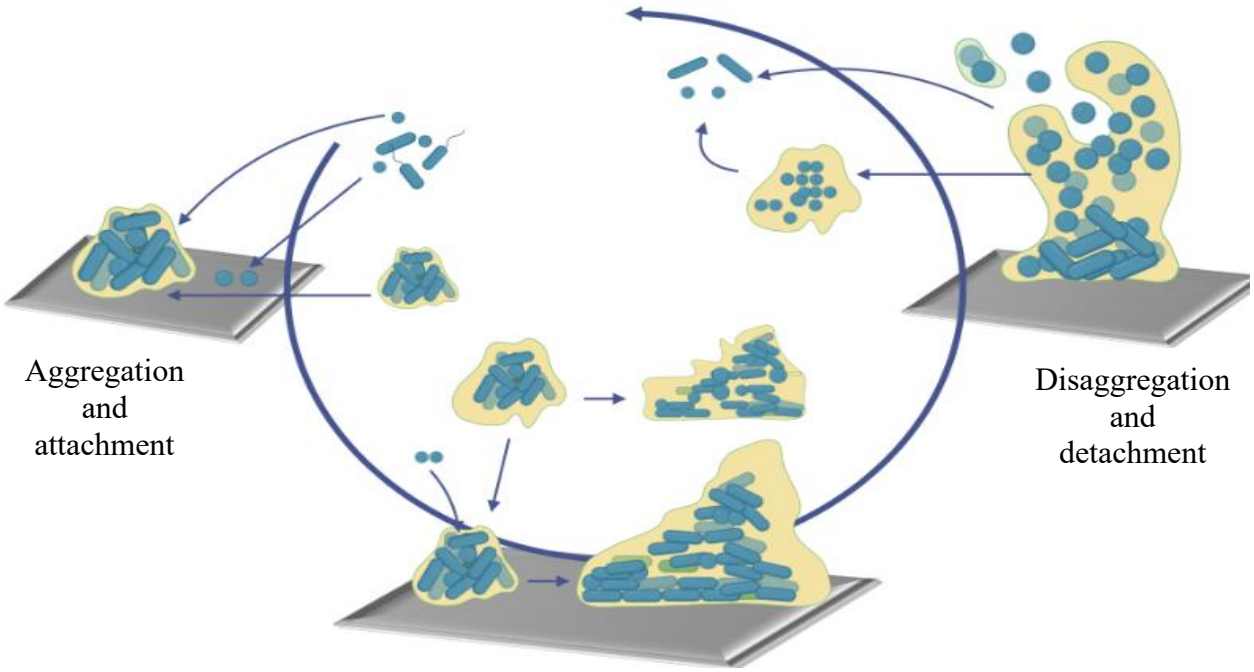


Figure 1.1. Stages of biofilm development. Biofilm formation proceeds through three main stages: (i) aggregation and attachment of planktonic cells to each other or attach to surfaces, (ii) growth and accumulation leading to maturation and matrix production, and (iii) disaggregation and detachment during which cells are released and colonize new sites. This conceptual model applies to biofilm development under in vitro, in situ, and in vivo conditions. Reproduced from (Sauer et al., 2022)

1.4 *Listeria* Biofilms in the Food Industry

The ability of *Listeria* to form biofilms is a major factor contributing to its persistence in the food processing environment, often leading to contamination of food products. Numerous outbreaks have been traced to cross-contamination originating from biotic or abiotic surfaces within processing facilities (Mazaheri et al., 2021). Biofilms act as major vehicles for transferring *L. monocytogenes* between food contact surfaces and products (Mazaheri et al., 2021; Ripolles-Avila et al., 2018). Inadequate cleaning allows these structures to establish and mature (Carrasco et al., 2012), while hard-to-clean niches, often resulting from poor sanitary design or equipment defects, can shelter cells, promote particle retention, and facilitate long-term colonization. Excessive wear and infrequent maintenance are additional factors that can contribute to contamination (Alves et al., 2024). Furthermore, extended production runs, mass product generation, and the structural complexity of facilities can increase the risk of biofilm formation by creating favorable conditions for microbial establishment (Olaimat et al., 2024).

Biofilm matrix enhances the likelihood of cell survival under unfavorable conditions, allowing them to persist in the environment for prolonged times (Colagiorgi et al., 2016). This matrix provides multiple protective benefits, including physical shielding, physiological adaptation, and enhanced intercellular communication, thereby hindering the impact of environmental stressors such as desiccation, nutrient depletion, oxidative stress, and UV radiation (Rather et al., 2021). Such multifaceted protection confers strategic advantage, increasing the

survival potential of *L. monocytogenes* in stressful environments (Mazaheri et al., 2021). Beyond these environmental stresses, the biofilm lifestyle plays a central role in reducing susceptibility to antimicrobial agents, contributing to transient tolerance that further complicates the control efforts. Mechanisms contributing to this tolerance include the reduced diffusion rates of antimicrobials within the biofilm matrix (Banerji et al., 2022), the presence of microenvironmental gradients that diminish antimicrobial efficacy, and the transition of a subpopulation of cells into a persister state (Rolon et al., 2024). Persisters exhibit temporary tolerance to antimicrobial agents, enabling survival under prolonged antimicrobial exposure (Wu et al., 2018). Importantly, once the stress is relieved, cells can revert to an active growth state, posing a further risk as a source of contamination. Stress induced changes in gene expression can also enhance tolerance (Wu et al., 2018). Tolerance in biofilms is therefore multifactorial: restricted penetration, biofilm thickness, reactivity of the antimicrobial within the matrix, concentration and mass transfer properties, and the physiological state of the cells all contribute to reduced efficacy (Wang, 2019).

Microorganisms within biofilms in food processing environments often comprise multiple species, including pathogenic and spoilage microorganisms, exhibiting an increased tolerance to antimicrobials (Brooks & Flint, 2008; Colagiorgi et al., 2017; Ripolles-Avila et al., 2018). The resident microbiota in these environments can affect the growth and survival of pathogens (Fagerlund et al., 2021). As an example, the presence of *Pseudomonas* spp., *Bacillus* spp. or *Acinetobacter* spp. can lead to the formation of robust biofilms, contributing to the enhanced survivability of *L. monocytogenes* (Rolon et al., 2024). This enhanced survival under antimicrobial pressure can occur through two complementary mechanisms: antimicrobial resistance, which is driven by resistance genes or mutations and reflected by increased minimum inhibitory (MIC) values, and antimicrobial tolerance, which allows cells to survive transient exposure (e.g.,

requiring longer contact time for a 2-log reduction) due to slow growth or reduced metabolic activity, even when MIC remains unchanged (Rolon et al., 2024). Several factors contribute to the increased resistance observed in multispecies biofilms compared to single species, such as genetic exchange, production of signaling molecules and metabolite exchanges (Ripolles-Avila et al., 2018). Beyond intrinsic defenses, microbial interactions within biofilms may further contribute to increased resistance. Mixed-species biofilms are structured in a way that ensures the fitness of the entire microbial consortium compared to single-species biofilms, which lack interspecies interactions. These spatial arrangements can lead to increased resistance to antimicrobial agents. The layering of bacteria can limit the access of the antimicrobial to cells colonizing the bottom layers (Rao et al., 2020). Moreover, cooperative interactions between different species can enhance adhesion of other species and create a favorable microenvironment for biofilm formation (Yuan et al., 2020). Rolon et al. (2024) compared the biofilms formed by *L. monocytogenes* alone and with *Pseudomonadaceae, Xanthomonadaceae, Microbacteriaceae*, and *Flavobacteriaceae,* showing that multispecies biofilms exhibited greater tolerance to benzalkonium chloride (Rolon et al., 2024). Similarly, Haddad et al. (2021) reported that *L. monocytogenes* had higher protection against desiccation benzalkonium chloride exposure when grown with *P. fluorescens.*

Several studies have demonstrated a strong association between *L. monocytogenes* and *Pseudomonas* spp. within biofilms in food processing environments (Puga et al., 2018). *Pseudomonas* spp. are spoilage microorganisms frequently isolated from surfaces in the food industry (Meesilp & Mesil, 2018), with many strains capable of efficiently producing biofilms and exhibiting resistance and tolerance following antimicrobial exposure (Thomassen et al., 2023). *Pseudomonas* was identified as the dominant bacterial group in three fruit processing plants where *L. monocytogenes* was also isolated (Fagerlund et al., 2021). This co-occurrence

between *Pseudomonas* and *Listeria* reflects a positive relationship, in which *Pseudomonas* can enhance the protection of *L. monocytogenes* against adverse environmental conditions, potentially benefiting from shared metabolic byproducts or from the structural advantages of joint biofilm formation (Fagerlund et al., 2021; Ripolles-Avila et al., 2018).

Rodriguez-Lopez et al. (2022) demonstrated that the architecture of *L. monocytogenes* biofilms on stainless steel is influenced by the presence of *Pseudomonas* spp., while Maggio et al. (2023) reported the frequent co-occurence of *L. monocytogenes* and *P. fluorescens* in dual-species biofilms in dairy plants. Puga et al. (2018) further showed that *L. monocytogenes* actively integrates into preformed *P. fluorescens* biofilms, preferentially colonizing the bottom layers, and that surfaces pre-colonized by *P. fluorescens* significantly promote *L. monocytogenes* attachment.

1.5 Strategies to Control *Listeria* Biofilms in the Food Industry: Cleaning and Sanitation

Listeria biofilms present a significant challenge in the food processing environment as they are more resistant and tolerant to common cleaning and sanitation strategies, thereby posing public health risks (Kannan et al., 2020; Liu et al., 2023b). Efforts have been directed toward developing effective methods to remove biofilms from surfaces (Carrascosa et al., 2021).

Cleaning refers to the physical removal of soil particles from surfaces by mechanical, manual, or chemical methods and it enhances the effectiveness of subsequent sanitation efforts (Quinn et al., 2015). The process begins with the removal of visible debris, followed by rinsing equipment with potable water, except for dry processing facilities. Any pooled water or drainage backups should also be eliminated to prevent a favorable environment for microbial persistence (Coughlan et al., 2016). Detergents are then applied to remove fat and protein residues, in which bacteria can survive and re-enter the system post-cleaning (Schmidt, 2012). These agents act by

neutralizing soils and helping removal through emulsification, dissolution, particle breakdown, and flushing (Etienne, 2006, p. 79). Cleaning compounds also lower the surface tension of water, allowing soil particles to loosen and preparing them for removal (Marriott & Gravani, 2006). Detergents may be applied as foams or aerosol sprays, with proper concentration, temperature, and contact time being critical considerations for their efficacy (Coughlan et al., 2016). Finally, a rinse step is performed before proceeding to sanitation (Schmidt, 2012). At this stage, the surfaces should be visibly clean (Coughlan et al., 2016).

Sanitation procedures are also critical for ensuring surface hygiene. Sanitation refers to reduction of microorganisms to safe levels and can be achieved through chemical and thermal treatments (Schmidt, 2012). Equipment is recommended to be sanitized immediately after cleaning to prevent recontamination (Etienne, 2006, pp. 84-89).

Commercial sanitizers can be applied in accordance with manufacturer's label instructions. Correct sanitizer concentration, contact time, and temperature are critical to achieve microbial reduction (Etienne, 2006, p. 84). In most cases, no potable water rinse is required following sanitization, unless specified on the product label instructions. Good Manufacturing Practices (GMPs) require thorough sanitation of all food contact surfaces (NSF International, 2021). Where equipment is used in a continuous production operation, surfaces must be properly cleaned and sanitized on a schedule that ensures microbial safety (Etienne, 2006).

1.6 Sanitizers

Several factors influence the effectiveness of a chemical sanitizer, such as sanitizer's pH, water hardness, and the initial microbial load (Schmidt, 2012). Since the sanitizing agent must be in direct contact with microorganisms to be effective, surface characteristics play an important role. Cracks, crevices, pits, and other irregularities can provide suitable environments that promote

microorganism survival, enabling biofilm formation and hindering sanitizer's effectiveness. In addition, the effectiveness of cleaning agents and sanitizers is influenced by multiple factors, including the composition of the biofilm, whether it is single species or mixed species, the type of surface colonized, and the environmental conditions under which the biofilm develops (Brooks & Flint, 2008; Maillard & Centeleghe, 2023). Several antimicrobial agents have been shown to exhibit limited ability to penetrate efficiently through the biofilm matrix (Grooters et al., 2024; Stewart, 2015). Previous studies have reported that *L. monocytogenes* biofilms exhibit greater resistance to sanitizing agents compared to free-floating cells (Pan et al., 2006). Chavant et al. observed reduced effect of quaternary ammonium compounds on biofilm cells compared to planktonic *L. monocytogenes* (Chavant et al., 2004). Similarly, Yun et al. (2012) demonstrated that *L. monocytogenes* biofilms were more resistant to hydrogen peroxide than planktonic cells (Yun et al., 2012). Rodriguez Lopez et al. (2018) attributed part of this increased resistance to physiological changes within biofilm cells, such as altered membrane fluidity, which reduces QACs effectiveness (Rodríguez-López et al., 2018).

Chemical sanitizers are typically categorized based on their mode of action. The most commonly applied in the food industry include chlorine-based compounds, iodine formulations, quaternary ammonium compounds (QACs), and oxidizing agents such as hydrogen peroxide and peracetic acid. Table 2 provides an overview of these sanitizers, including their mode of action. Each class differs in activity, stability, and compatibility with surfaces.

Oxidizing agents damage *L. monocytogenes* by disrupting the cell wall and membrane, denaturing proteins, and nucleic acids. In response, the bacterium senses stress and activates protective pathways, including the σ^B regulon, cold and heat shock proteins (*cspABCD*), proteases (*clpC, clpP, groEL*), and oxidative stress genes such as *sod*, *perR*, and *kat* (Manso et al., 2020). The

general stress response is coordinated by the alternative sigma factor (σ^B), which positively regulates over 300 genes involved in stress adaptation, virulence, and metabolic activity (Arvaniti et al., 2025). Collectively, these mechanisms enable *L. monocytogenes* to withstand hostile environmental conditions. In addition, chlorine, one of the most widely used sanitizers in the food industry, has been shown to induce a viable but non-culturable (VBNC) state in *L. monocytogenes*, reducing the effectiveness of disinfection and allowing the pathogen to persist undetected while retaining the ability to cause disease (Banerji et al., 2022).

Table 1.2. Characteristics of some commonly used sanitizers

Sanitizer	**Typical Concentrations for food contact surfaces**	**Optimum pH**	**Effect of water hardness**	**Mode of action**
Chlorine solutions	50-200 ppm	6.5 - 7	Activity decreases in very hard water	Free chlorine can cause: -oxidation of sulfhydryl enzymes and amino acids. -ring chlorination of amino acids -loss of intracellular contents -inhibition of protein synthesis -decreased ATP production (CDC, 2023)
Peracetic acid	150-200	5	Stable across hardness levels	- denaturation of proteins - disrupt cell wall permeability - oxidation of sulfhydryl and sulfur bonds in proteins, enzymes, and other metabolites (CDC, 2023)

Silver dihydrogen citrate	20-30 ppm	~ 7	Stable across hardness levels	- Interaction of silver ions with thiol groups in enzymes and proteins. - Disrupting DNA replication (Pure BioScience Inc., 2016)
Lactic acid	1-4%	3 - 4	Stable across hardness levels	- Acidification of the cytoplasm through undissociated molecules, leading to deformation and damage to enzymatic activities, proteins, and DNA structure. - Leakage of cellular components due to membrane damage. - Changes in cell membrane permeability, hindering substrate transport (Alakomi et al., 2000).

1.6.1 Chlorine compounds

Chlorine is one of the most widely used sanitizers in the food industry, available in various forms, including liquid chlorine, hypochlorites, inorganic and organic chloramines, and chlorine dioxide (Marriott & Gravani, 2006; Schmidt, 2012). Among these, liquid chlorine, a solution of sodium hypochlorite (NaOCl) in water, is the most commonly used form (CDC, 2023). When chlorine is added to water, it reacts to form dissolved compounds with antimicrobial activity (CDC, 2023). The microbicidal activity of chlorine is largely attributed to undissociated hypochlorous acid (HOCl), while its dissociation to the less active hypochlorite ion (OCl^-) depends on the pH of the solution (CDC, 2023). However, the use of high chlorine concentrations increases the risk of forming potentially hazardous by-products (Virto et al., 2005).

The efficacy of chlorine is strongly influenced by pH and temperature. At higher pH, hypochlorous acid shifts toward the less potent hypochlorite ion, thereby reducing its disinfecting

power (CDC, 2023). In addition, sodium hypochlorite decomposes over time, necessitating regular monitoring to maintain the desired available chlorine concentration. The presence of organic matter is also critical, as it readily reacts with and inactivates chlorine (Etienne, 2006; CDC, 2023).

Several studies have demonstrated the effectiveness of liquid chlorine in reducing the microbial contamination on food contact surfaces (Byun et al., 2021; Hua et al., 2019; Maifreni et al., 2023; H. Wang et al., 2018). Byun et al. (2021) studied the efficacy of sodium hypochlorite against *Salmonella* Enteritidis biofilms, observing greater reductions as the concentration increased from 10 to 100 ppm, with the highest reduction on stainless steel (Byun et al., 2021). Similarly, Hua et al. (2019) reported that the treatment of stainless steel, rubber, and LDPE surfaces with 200 ppm chlorine for 5 min achieved approximately a 3-log CFU/coupon reduction in *L. monocytogenes* biofilms (Hua et al., 2019). However, Behnke et al. (2011) emphasized that chlorine susceptibility may vary depending on biofilm community composition, with multispecies biofilms often exhibiting greater tolerance compared to monospecies communities (Behnke et al., 2011).

1.6.2 Peroxides

Peroxides are compounds that contain at least one pair of covalently bounded oxygen atoms and are broadly classified into inorganic and organic groups. Hydrogen peroxide, an inorganic peroxide, functions primarily as an oxidizing agent. Peracetic acid, one of the most commonly used antimicrobials in the food industry, belongs to the organic peroxide group and is characterized by low pH and a vinegar-like odor (Ao et al., 2021; Stearns et al., 2022). PAA has been registered in the U.S. as a sanitizer and pesticide since 1985 and is produced through the equilibrium reaction of acetic acid and hydrogen peroxide (Arvaniti et al., 2025; Petri et al., 2021). Its antimicrobial activity is attributed to strong oxidizing capacity, where it generated free radicals upon contact

with organic compounds. Its mode of action involves oxidizing sulfhydryl and sulfur bonds, damaging metabolic enzymes and disrupting cell membrane integrity (USDA, 2000; Aalto et al., 2024).

PAA has been extensively studied for its antimicrobial performance on both foods and food contact surfaces (Hua et al., 2019; Korany et al., 2018; Poimenidou et al., 2016). While PAA is highly effective against both Gram-positive and Gram-negative bacteria, its efficacy against biofilms can vary depending on biofilm structure and composition (Goetz et al., 2022). Hua et al. (2019) reported that PAA was the most effective sanitizer compared to chlorine and QACs against *L. monocytogenes* biofilms on food contact surfaces, although its performance was limited by the presence of organic matter (Hua et al., 2019). Similarly, Poimenidou et al. (2016) observed an average of 2.8 log CFU/cm^2 reduction on polystyrene following a 5 min exposure to 2000 ppm PAA.

1.6.3 Lactic acid

Lactic acid is a naturally occurring substance that exhibits antimicrobial activity against foodborne pathogens, and its applications in decontamination of various food products have been studied (Kostoglou et al., 2024; Lindsay et al., 2022; Nkosi et al., 2021; Stanojević-Nikolić et al., 2016). The antimicrobial mechanism of lactic acid is primarily attributed to its undissociated form, which penetrates microbial cell membranes and lower intracellular pH. This acidity disrupts enzymatic activities, damages proteins, DNA structure, and ultimately leads to cell death (Stanojević-Nikolić et al., 2016).

Several studies have evaluated the use of lactic acid for surface decontamination (Ban et al., 2012; Mendez et al., 2022; Yang et al., 2009). Yang et al. reported that lactic acid-based sanitizer (0.18%) application for 5 min effectively reduced seven-day-old *L. monocytogenes*

biofilms formed on both rough and smooth HDPE surfaces (Yang et al., 2009). Ban et al. (2012) observed a 0.94 log CFU/coupon reduction on PVC surfaces following 30 s immersion treatment with lactic acid at 2% (Ban et al., 2012). Similarly, Mendez et al. (2022) demonstrated significant reductions when applying 4% lactic acid for 10 min against *L. monocytogenes* biofilms on stainless steel (Mendez et al., 2022).

1.6.4 Silver Dihydrogen Citrate

Silver ions are well-known for their antimicrobial effects against microorganisms and have been used in a variety of industries (Jo et al., 2007; Sim et al., 2018). The antimicrobial activity of silver ions is primarily attributed to their interaction with thiol groups in proteins (Jo et al., 2007; Jung et al., 2008). Another mechanism involves interference with DNA, including inhibition of DNA replication through enhanced pyrimidine dimerization (Jo et al., 2007).

Silver's antimicrobial properties are further enhanced when combined with citric acid to form silver dihydrogen citrate (SDC), which improves stability (Buckley et al., 2018; Manuel et al., 2017). Bacteria uptake SDC as a potential nutrient source, allowing silver ions to enter the cell, where they interfere with DNA and inhibit replication. Another mode of action is the inactivation of proteins, ultimately leading to cell death (PURE Bioscience Inc, 2016). Masuku et al. (2014) demonstrated the effectiveness of SDC in reducing *Listeria* populations on deli meat slicers by 4.87 and 5.63 logs. Ivers et al. (2024) demonstrated its efficacy against *Salmonella* biofilms on various surfaces grown in a CDC biofilm reactor, reporting a 2.8 log CFU/coupon reduction on HDPE coupons after 1 min of exposure. Additionally, Manuel et al. (2017) highlighted the efficacy of SDC as a surface disinfectant against norovirus.

1.6.5 Ultraviolet Irradiation

The impact of ultraviolet (UV) irradiation has been widely studied on biofilms (Bernbom et al., 2011; Bintsis et al., 2000; Torkzadeh & Cates, 2021; Z. Zhao et al., 2023). UV radiation spans wavelengths 100-400 nm and is categorized into three types: UVA (315-400 nm), UVB (280-315 nm), and UVC (100-280 nm) (CDC, 2025). Of these, UVC light at 254 nm is the most commonly applied for disinfecting air, water, and surfaces due to its germicidal activity (A. R. Pereira et al., 2023).

The antimicrobial effect of UVC arises from its ability to damage nucleic acid of microorganisms (Reed, 2010). DNA and RNA bases absorb UVC photons, resulting in the formation of photoproducts such as cyclobutene pyrimidine dimers (CPDs). These lesions prevent accurate base pairing and interfere with DNA replication and transcription, ultimately leading to cell death (Ploydaeng et al., 2021).

Numerous studies have demonstrated UVC's effectiveness against biofilms on food contact surfaces. Bernbom et al. (2011) reported significant inactivation of *L. monocytogenes* biofilms exposed to UVC, while Green et al. (2020) showed strong reductions on stainless steel surfaces. Jung et al. (2008) highlighted UVC efficacy on fresh produce, suggesting its potential for both surface and food applications. More recently, Méndez et al. (2022) found UVC treatment effective against mature biofilms, though efficiency decreased with increased surface roughness and shading effects (Bernbom et al., 2011; Green et al., 2020; Jung et al., 2008; Mendez et al., 2022).

1.7 Hurdle Technologies

Hurdle technology refers to a combined use of multiple preservation methods to inactivate or inhibit microorganisms while maintaining food quality and stability (Putnik et al., 2020). The principle is based on creating an environment that is unfavorable for microbial survival, where

each “hurdle” (e.g., pH, temperature, water activity, pressure, radiation, or antimicrobial compounds) imposes a specific stress that forces microorganisms to expend energy to adapt (Putnik et al., 2020). When several hurdles are applied at mild intensities, they often exhibit synergistic inhibition or inactivation of microorganisms, while minimizing negative effects on sensory and nutritional quality (Mukhopadhyay & Gorris, 2014; Fellows, 2009; Khan et al., 2017).

Combining two or more interventions enhances efficacy because each hurdle targets microbes through a different antimicrobial mechanism. Several studies have demonstrated the effectiveness of hurdle technology for biofilm control (Ban et al., 2012; Ghahari & Khosravi-Darani, 2024; Kim et al., 2016; Mendez et al., 2022; Yuan et al., 2021). For example, Ban et al. (2012) showed that combining a sanitizer with steam achieved up to a 2.78 log CFU/coupon reduction of *E. coli*, *S. Typhimurium*, and *L. monocytogenes* biofilms on stainless steel, which was greater than either treatment alone (Ban et al., 2012). Similarly, Masuku et al. (2014) reported improved inactivation of adhered *Listeria* spp. on cast aluminum and stainless steel surfaces when SDC was combined with moist heat (Masuku et al., 2014). Kim et al. (2016) also demonstrated enhanced reductions of foodborne pathogens when ethanol and UV treatments were applied together (Kim et al., 2016).

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Chapter 2 - Knowledge Gap and Research Objectives

L. monocytogenes is a significant food safety concern, posing risks to public health and creating challenges for the food industry. *L. monocytogenes*' ability to form biofilms enables its persistence in food processing environments, whereby contamination of food processing equipment can lead to subsequent contamination of food products. Within biofilms, *L. monocytogenes* cells are protected from adverse environmental conditions and exhibit increased tolerance to antimicrobials, thereby compromising sanitation efforts. Understanding the formation of *L. monocytogenes* biofilms on various surfaces commonly used in the food industry is essential to improve control strategies, as surface characteristics of materials influence biofilm formation and sanitizer efficacy. In addition to surface properties, the mode of action of specific sanitizers plays a critical role in biofilm control by influencing gene expression. Variations in gene expression profiles reflect differences in the kinetic antimicrobial action, highlighting the need to study the transcriptomic response of *L. monocytogenes* within biofilms. Furthermore, *L. monocytogenes* often coexists with other species, such as *Pseudomonas* in food processing environments, forming dual and multi species biofilms with enhanced resistance and tolerance to environmental stresses due to complex interspecies interactions. While various antimicrobial treatments have been studied to control biofilms, limited research has explored and compared the use of hurdle technology (application of more than one antimicrobial strategy sequentially or simultaneously) against both single and dual species biofilms. Therefore, the objectives of this research were to:

- Assess the growth of *L. monocytogenes* biofilms on different surfaces representative of food processing facilities and evaluate the efficacy of commercially available sanitizers and UV-C light alone or simultaneously.

- Characterize transcriptional responses and survival of *L. monocytogenes biofilms* exposed to sublethal concentrations of various commercial sanitizers and elucidate mechanisms that may contribute to biofilm adaptation, survival, and tolerance.
- Investigate the dynamic population interactions between *Pseudomonas fluorescens* and *L. monocytogenes* when forming dual-species biofilms.
- Evaluate the efficacy of commercially available sanitizers in controlling growth and formation of *P. fluorescens* and *L. monocytogenes* dual-species biofilms on different food-contact surfaces.

Chapter 3 - Formation and control of *Listeria monocytogenes* growth and accumulation on food processing surfaces in the fresh produce industry (published in Journal of Food Protection)

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Abstract

The ability of *Listeria monocytogenes,* a common foodborne pathogen, to form biofilms significantly contributes to its persistence and contamination risks. Understanding *L. monocytogenes* aggregation is crucial to develop effective control strategies. This study assessed the growth and accumulation of *L. monocytogenes* on common food processing surfaces in the produce industry and evaluated the efficacy of commercially available sanitizers alone or simultaneously with UV-C light for 30 sec, 1 or 5 min. *L. monocytogenes* was allowed to aggregate on stainless steel, nylon, high-density polyethylene (HDPE), polyvinyl chloride (PVC), and Teflon for 24 h (initial biofilm aggregate) and 96 h (mature biofilms). Treatments included 120 ppm peracetic acid (PAA), silver dihydrogen citrate (SDC), 4% lactic acid, and UV-C light (254 nm). After sanitizer application, cells were dislodged and enumerated by serial dilution and plating. Confocal images were obtained before and after treatments to evaluate biofilm architecture. All liquid sanitizers significantly reduced *L. monocytogenes* independently of the surface type ($P < 0.05$). UV-C treatment application time had no significant effect on biofilm reduction ($P > 0.05$), though its efficacy on 24 h aggregates varied significantly depending on the surface material. Peracetic acid and lactic acid were the most effective treatment across surfaces, while UV-C alone was the least effective. Combined sanitizer and UV-C light application for up to 5 min did not necessarily improve treatment efficacy compared to sanitizers alone. Confocal images displayed surface cleanability and alterations in microbial architecture before and after treatments. Mature biofilms exhibited extensive surface coverage on all surfaces, which was visibly reduced following sanitizer application. This study provides insightful information to the produce industry for using sanitizing approaches on various surfaces based on cleanability, topographical and architectural characteristics.

Introduction

Since 2011, *Listeria monocytogenes* has been a growing food safety concern in the fresh produce industry, including the organic market (Estrada et al., 2020; Townsend et al., 2021). It has been linked to several outbreaks and recalls due to the contamination of produce, largely because a significant amount of fresh produce is consumed raw, increasing the potential risk of transmission to humans (Angelo et al., 2017; Chen et al., 2016; Maffei et al., 2016; Nyarko et al., 2018; Steele & Odumeru, 2004; FDA, 2025). *L. monocytogenes* is among the leading causes of foodborne illness-related deaths in the USA, responsible for 1,600 cases every year, with a 16% mortality rate, far higher than other produce-associated foodborne pathogens like *Salmonella* and Shiga-toxigenic *Escherichia coli* (CDC, 2024a; Marshall et al., 2020; Scallan et al., 2011). In 2024, the Centers for Disease Control and Prevention (CDC) reported a multistate outbreak of *L. monocytogenes* linked to peaches, nectarines, and plums, resulting in one fatality (CDC, 2024b). Concurrently, another *Listeria* outbreak linked to leafy greens highlighted the ongoing risks of this pathogen in the fresh produce (CDC, 2024c).

L. monocytogenes causes an infection called listeriosis in high-risk groups such as pregnant women, people with weakened immune systems, and the elderly through the consumption of contaminated food (Nowak et al., 2021; Pang et al., 2019). *L. monocytogenes* is widely spread in nature and well-adapted to life in mammalian cells as well as in soil (Freitag et al., 2009). Given this, *L. monocytogenes* can be isolated from various sources in the environment, and contamination of food products can occur at any stage of the produce supply chain (Estrada et al., 2020). Nevertheless, several studies have shown that fresh produce is most often contaminated during post-harvest handling and processing (Nyarko et al., 2018; Ruiz-Llacsahuanga et al., 2021; Self et al., 2019; Sullivan & Wiedmann, 2020), therefore sanitation strategies for food-contact surfaces

are important to prevent the spread of this pathogen. Food-contact surfaces in fresh produce facilities undergo daily cleaning and sanitation using a variety of chemical sanitizers, such as chlorine, peroxyacetic acid (PAA), chlorine dioxide, sodium chlorite, or organic acids depending on the type of operation, such as organic or conventional (Hua et al., 2019; Truchado et al., 2021). Organic producers in the U.S. can only utilize synthetic sanitizers that are listed in the "National List of Allowed and Prohibited Substances" (USDA - Agricultural Marketing Service, 2024) to maintain organic certification, with notable absences of regularly used sanitizers like quaternary ammonia compounds. Therefore, organic growers have fewer options available to control foodborne pathogens as compared to conventional production.

Studies reported that the efficacy of the sanitizers was influenced by the type and characteristics of surface materials (Hua et al., 2019; Ivers et al., 2024; Oh et al., 2025; Stewart et al., 2025; Yuan et al., 2021). Difficult-to-clean sites in equipment used in the field and processing facilities, such as connecting points, welding joints, and corners, require particular attention during cleaning and sanitation as they are prone to fouling, thus facilitating bacterial aggregation (Pan et al., 2006). Furthermore, the use of chemical sanitizers alone is not always sufficient to control microbial contamination on surfaces (Hua et al., 2019; Korany et al., 2018).

L. monocytogenes can aggregate and form biofilms, allowing it to survive under adverse environmental conditions such as harsh temperatures, nutrient depletion and pH fluctuations (Azizoglu et al., 2015; Blackman & Frank, 1996; Lee et al., 2019; Nowak et al., 2021). An updated biofilm model proposed by Sauer et al. (2022) outlines three main events in biofilm formation, encompassing diverse habitats and microenvironments, including open systems. A first phase involves aggregation and attachment of cells, where bacteria aggregate with each other or attach to biotic or abiotic surfaces; a subsequent phase where the aggregated and attached colonies expand

through growth and produce extracellular polymeric substances (EPS); and finally, a disaggregation and detachment event where bacteria can leave the biofilm as aggregates and/or as single cells (Sauer et al., 2022).

Limited data are available on the growth and accumulation of *L. monocytogenes* on different food contact surfaces found in the produce industry such as Teflon, polyvinyl chloride (PVC), and high-density polyethylene (HDPE). In our study we aimed to evaluate the efficacy of the commercially available sanitizers 120 ppm PAA, 4% (v/v) lactic acid, and silver dihydrogen citrate (SDC), alone or simultaneously applied with UV-C light in controlling *L. monocytogenes* growth and accumulation for 24 h (biofilm-like aggregate) and 96 h (mature biofilms) on surfaces representative of the fresh produce industry.

Materials and Methods

Bacterial strains. Three strains of *L. monocytogenes* were used in this study: 390-1 (serotype 1/2a), 390-2 (serotype 1/2b) both environmental isolates from the 2011 cantaloupe outbreak (CDC, 2012); and strain 573-035 (serotype 4b) clinical isolate from the 2014-2015 caramel apple outbreak (CDC, 2015) were selected. All the isolates were provided by the Department of Food Science and Technology at the University of Georgia. The strains were stored at -80°C in CryoCare Bacteria Preserver beads (Key Scientific, Stamford, TX, USA) until use.

Coupons material. Five different materials representatives of food contact surfaces commonly encountered in the fresh produce industry were used in this study: stainless steel grade 304, high-density polyethylene (HDPE), nylon, polyvinyl chloride (PVC), and Teflon®. For 24 h aggregate studies, all material sheets were acquired from W. W. Grainger Industrial Supply (Lake Forest, IL) and cut into coupons of 2.5 cm by 5 cm. For biofilm studies, stainless steel 304, HDPE, and nylon disc coupons (1.27 cm in diameter) were purchased from BioSurface Technologies

Corporation (Bozeman, MT, USA). Teflon and PVC materials were obtained from Sparks Belting (Grand Rapids, MI, USA) and cut into 1.27 cm in diameter by a Bodor P3015T Fiber Laser cutting machine located at the Technology Development Institute at Kansas State University. Disc coupons were used to fit within the rods of the biofilm reactor. For biofilm experiments, all coupons were sterilized at 121℃ for 15 min followed by cleaning steps: scrubbing with soapy water, sonication (Branson 3800) in soapy water for 5 min, sonication in tap water for 5 min, and finally sonication in deionized water for 5 min. Coupons were then dried under UV-C light for 15 min. Stainless steel and Teflon coupons were sterilized at 121 ℃ for 15 min to remove background microflora. Coupons that were high-temperature sensitive were used as new and washed with Tergazyme (Alconox Inc., White Plains, NY), rinsed with water, and air dried.

24 h biofilm-like aggregate formation. The method described by Ivers et al. (2024) was used to grow 24 h aggregate *L. monocytogenes* cells with some modifications. Prior to use, 10 µl of each strain was grown in 10 ml of tryptic soy broth with yeast extract (0.6% w/v; TSBYE; Difco, Becton Dickinson Co., Sparks, MD) at 35°C for 24 h. Three successive transfers at 24 h intervals each was performed for each strain on TSBYE. After the third transfer, 250 µl of overnight culture was spread plated onto tryptic soy agar with yeast extract (0.6% w/v) (TSAYE; Difco, Becton Dickinson Co., Sparks, MD) and incubated at 35°C for 24 h to create a bacterial lawn. The bacterial lawn was harvested by adding 10 ml of buffered peptone water (BPW; Difco, Becton Dickinson Co., Sparks, MD) to each plate, and scraped using a sterile spreader. Each harvested lawn was combined in a sterile centrifuge tube to create a three-strain cocktail at a concentration of 9.5 log CFU/ml. The cocktail was diluted in 0.1% (w/v) peptone water (Difco, Becton Dickinson Co., Sparks, MD) to verify its initial concentration by spiral plating on modified Oxford medium (MOX; Difco, Becton Dickinson Co., Sparks, MD) containing modified Oxford

antimicrobic supplement (Bacto, Becton Dickinson Co., Sparks, MD) and incubated at 35°C for 48h.

Coupons were inoculated using a spot inoculation method. Surfaces were inoculated by spotting 100 µl of the *L. monocytogenes* cocktail (9 log CFU/ml), distributed in ten 10 µl drops over the surface area. Surfaces were held in a biosafety cabinet at room temperature (22°C) for 2 h to dry the inoculum. For each treatment and material type, coupons were inoculated with three independently grown *L. monocytogenes* cocktails (three biological replicates), with two technical replicates each (n=6).

Mature biofilm growth. Bacteria strains were streaked onto Tryptic Soy Agar (TSA, BD Difco, Sparks, MD, USA) for isolation and incubated at 37 ± 2 ° C for 24 h. For each strain, one isolated colony was used to inoculate 10 ml of fresh Tryptic Soy Broth (TSB, BD Difco, Sparks, MD, USA) for 24 h at 37± 2 °C with 70 rpm shaking. A multi-strain cocktail of *L. monocytogenes* strains was prepared by combining 1 ml aliquots from each bacterial suspension. The initial cocktail population was verified by serial dilution and spread plating on TSA (TSA, BD Difco, Sparks, MD, USA).

A CDC Biofilm Reactor was used to grow mature biofilms for 96 h operated in a fed-batch operation described as follows. An aliquot (1 ml) of *L. monocytogenes* cocktail (average initial population of 8.99 log CFU/ml) was used to inoculate a vessel containing 350 ml of sterilized TSB prepared using deionized water (at a concentration of 20 g/l). The reactor was maintained at batch conditions for 24 h at 22 ± 2°C with baffle stirring at 60 rpm as previously described (Manville et al., 2023a). During the continuous phase, nutrients (sterilized TSB at a concentration of 1g/l) were constantly supplied to the system at a flow rate of 5 ml/min for 72 h. Biofilms were allowed to

develop on both sides of the coupons. Coupon positions were randomized within bioreactor rods prior to reactor assembly to minimize positional bias across replicates.

Assessment of accumulation and biofilm production by crystal violet staining. To assess the ability of all isolates used in the study to accumulate and produce biofilms, an established staining protocol was followed (Di Ciccio et al., 2022). Overnight cultures in TSB were centrifuged at 4000 rpm for 10 min, and the resulting pellets were resuspended and diluted to achieve a final inoculum concentration of 10^6 CFU/ml. This suspension was directly dispensed into each well of a sterile 96-well polystyrene microplate. TSB was used as a negative control. The well plate was incubated statically for 24 h at 37°C. After incubation, the contents of the well plate were discarded and 100 µl PBS was added to each well. This washing step was repeated three times. Subsequently, plates were incubated at 60°C for 1 h to fix the cells. To stain the residual bacterial biomass, 75 µl of 0.2% w/v crystal violet solution was added to each well and the plate incubated for 15 min at room temperature. The contents of the wells were removed by tapping the plate upside down. All the wells were then washed with sterile distilled water three times. When the plate was completely dry, 75 µl of 95% ethanol solution was added to each well and allowed to sit for 15 min. The absorbance was measured at 600 nm using a microplate reader (BioTek, Epoch 2, VT, USA). The results were interpreted based on the classification system by Stepanović et al. (2000). The Abs-600 nm cut-off for the negative control was calculated by using the mean Abs-600 nm of all negative control (NC) wells plus three standard deviations (Abs NC). The strains were then categorized as weak (Abs NC< Abs-600 nm ≤ 2× Abs NC), moderate (2 × AbsNC < Abs-600 nm ≤ 4 × AbsNC) or strong (4 × AbsNC < Abs-600 nm). All strains were tested in triplicate and the results were averaged.

Biofilm attachment. *L. monocytogenes* biofilms were grown for 96 h using a CDC Biofilm Reactor as described above and evaluated for their attachment through a series of rinsing steps at sampling times (2 h, 24 h, and 96 h) as described by (Manville et al., 2023a). Briefly, after 2 h, coupons were aseptically removed from the CDC Biofilm reactor and placed in a beaker containing 50 ml of sterile deionized water for 10 s. Next, coupons were transferred to another beaker for 10 s, and this rinse step was repeated one more time to remove loosely attached cells. Coupons were allowed to dry for 30 min and then strongly attached cells were recovered by sonication for 30 s at 40 kHz (Branson 3800, USA) and vortexing for 30 s in 10 ml of PBS. This procedure was repeated three times to ensure complete detachment (ASTM, 2019). Serial dilutions were performed and plated in duplicates on TSA. Uninoculated coupons were considered negative controls.

Antimicrobial applications. The efficacy of three commercially available sanitizers applied alone or simultaneously with UV-C light was evaluated on 24 h biofilm-like aggregate and 96 h mature biofilms: Silver Dihydrogen Citrate containing 4.85% citric acid and 0.003% silver ions (PURE Bioscience, El Cajon, CA, USA) as per label instructions, 4% lactic acid solution (Purac® Corbion, Blair, NE), measured using a lactic acid test kit (ChemWorld, Kennesaw, GA, USA) and 120 ppm Peracetic Acid (SaniDate 15.0, BioSafe Systems, LLC, East Hartford, CT), confirmed using a peracetic acid test kit (ChemWorld, Kennesaw, GA; AquaPhoenix Scientific, Hanover, PA). A UV-C lab bench setup was designed and built in an enclosed box system (Figure 3.1) to simulate commercial conditions and provide consistent irradiation and uniform application of the sanitizers. This type of system effectively prevented the spread of pathogenic microorganisms when spraying and allowed for collection of effluent in a disposal bin for subsequent autoclaving. A germicidal UV lamp emitting UV-C light (254 nm) was used as a light

source. The intensity of the UV-C light was monitored using a UV-C light meter (ILT2400 UVGI-NB, International Light, MA; Analytik Jena US, Upland, CA). The UV-C lamp was kept 16 cm away from the coupons with an intensity range of 3.16- 4.50 mW/cm^2 (corresponding to a UV dosage between 948 and 1350 mWs/cm^2). Sanitizers were applied with a steady flow rate of 400 ml/min through a pump feeding into a food-grade nozzle that ensures all coupons were treated. For 24 h aggregate experiments, the sanitizers were applied for 30 sec or 1 min without coupon rotation. For mature biofilms, sanitizers were applied for 1 min or 5 min, where the sanitizer was sprayed for 1 min and allowed to sit on the coupons until end of the application time (5 min). For both 1 min and 5 min treatments, the rods were rotated halfway through spraying to ensure all surfaces received equal exposure. For the simultaneous application of the UV-C and the sanitizers, both the UV-C light and sanitizer pump were on for the specified time. Preliminary experiments showed a low efficacy of the UV-C light when applied for 1 min, therefore UV-C light was applied for both experiments during 5 min by maintaining the UV-C light on for the full 5 min.

Sanitizer treatments, concentrations, and application times were determined based on industry needs, and Environmental Protection Agency (EPA) label guidelines. Options for both organic and conventional fresh produce operations included peracetic acid (PAA), lactic acid, and UV-C light. Although silver dihydrogen citrate (SDC) is currently not approved for organic farming, it was chosen for its ready-to-use formulation, which reduces the risk of manual preparation errors. U.S. regulations have stated that lactic acid antimicrobial end-use product can range from 0.16% to 17.29% (EPA, 2021).

Recovery and enumeration of 24 h biofilm-like aggregates. After treatment, each coupon was placed into a 50 ml conical centrifuge tube containing 30 ml of Dey/Engley Neutralizing broth (Neogen, Lansing, MI), to arrest sanitizer activity. Samples were vortexed for

30 s to remove remaining cells and serially diluted in 0.1% peptone water (Bacto, Sparks, MD, USA) to enumerate *L. monocytogenes* populations. One hundred microliters were spiral plated in duplicates onto MOX plates and incubated at 35°C for 48 h. When the population counts fell below the limit of detection (2.50 log CFU/surface), the standard value of 2.44 log CFU/surface was assigned for calculations.

Recovery and enumeration of mature biofilms. After treatment, coupons were individually transferred into 10 ml of Dey Engley Neutralizing Broth (Hardy Diagnostics, Santa Maria, CA, USA), while control (non-treated coupons) and UV-C treated coupons were placed in 10 ml of Phosphate-Buffered Saline (PBS, VWR, Solon, OH, USA). Samples were sonicated for 30 s and vortexed for 30 s, repeating three times to ensure the full detachment and recovery of the cells (ASTM, 2019). Serial dilutions were performed in 0.1% peptone water (Bacto, Sparks, MD, USA) and spread plated on MOX in duplicate. Plates were incubated at 37°C for 24 h and results were recorded and presented as log CFU/coupon.

Microscopy analysis. To visualize surface topography, clean coupons (without cell aggregate) were individually mounted on carbon tape on top of aluminum stubs. Except for stainless steel samples, all coupons were coated with 10 nm gold using the SPI-Module Sputter Coater (SPI supplies, West Chester, PA). Samples were imaged using a S4700 II Cold Field Emission Scanning Electron Microscope (SEM, Hitachi High Technologies) located at the Microscopy Facility of the University of Kansas with lower and upper secondary electron detectors. The data were acquired with the following settings: Ultra-High-Resolution operation mode, condenser lens at 3, 5kV accelerating voltage, 7-10 uA emission current, aperture 2 (50 µm diameter), 8.0 ±1 mm working distance, and at 2560 x 1920 pixels resolution with 40-80s scan speed.

Mature biofilms before and after treatments on different coupon materials were instead analyzed using Laser Scanning Confocal Microscopy (LSCM) (LSCM-5 Pascal associated with a Zeiss Axioplan 2) located at the Microscopy Facility of the Division of Biology at Kansas State University. To visualize the live and dead cells within the biofilms, SYTO 9 was used to stain live cells, and SYTOX^{TM} Red was used for dead cells (dyes obtained from ThermoFisher Scientific, Eugene, OR) (Manville et al., 2023a). All images were processed using ImageJ2 (NIH, USA).

Experimental design and statistical analysis. The different treatment effects were studied in an experiment with a 5x7x2 factorial treatment structure with a control, implemented within an incomplete split-plot design with three replicates. The experiment involved five materials: stainless steel 304, HDPE, nylon, Teflon, and PVC. Seven different treatments and a control were applied to these materials, with two different antimicrobial exposure times [24 h aggregate study (30 s and 1 min) and for mature biofilm study (1 and 5 min)]. The treatments included the combination of three chemical sanitizers and the presence or absence of UV-C light. Each treatment was randomized across the eight rods in the CDC bioreactor. The data were analyzed using a linear mixed effects model including material, sanitizer treatment, time, and their interactions as fixed effects, the random effect of the CDC bioreactor, and the random effect of the sanitizer treatment nested in the CDC bioreactor. To evaluate differences in the results, an analysis of variance and post-hoc multiple comparison of means tests was implemented, using Tukey adjustments with a significance level of 0.05. For individual comparisons between exposure times, simple mean contrasts were implemented. Note that multiple comparisons using Tukey adjustments may be more conservative in determining significant differences between means (Table 1). All statistical analyses were performed using R (version 4.4.0, R Foundation for Statistical Computing, Vienna, Austria).

Results

Ability of strains to accumulate and produce biofilms. All *L. monocytogenes* strains used in this study were capable of forming biofilms, with varying levels of biofilm production as measured by crystal violet staining (Figure 3.2). The results of this assay classified *L. monocytogenes* 390-1 (serotype 1/2a) and *L. monocytogenes* 390-2 (serotype 1/2b) as moderate biofilm producers, whereas *L. monocytogenes* 573-035 (serotype 4b) exhibited weak biofilm production based on criteria established by Stepanović et al. (2000). Strains with greater accumulation and biofilm production abilities may be more challenging to control in processing environment, potentially complicating sanitation strategies and leading to product contamination. Biofilm forming ability may influence persistence in processing environments, though previous studies have reported conflicting results (Di Ciccio et al., 2022).

Cell attachment to the different surfaces. The attachment dynamics observed across different surfaces and times are presented in Figure 3.3. *L. monocytogenes* attachment was significantly affected by surface type and attachment time ($P < 0.05$). After 2 h of incubation, Teflon and PVC showed higher aggregated populations compared to nylon, stainless steel, and HDPE surfaces ($P < 0.05$), where counts were below the detectable limit (< 1.95 log CFU/coupon). After 24 h, attached cell populations significantly increased on all surfaces compared to 2 h ($P < 0.05$), with Teflon and PVC reaching 8.02 ± 0.36 and 8.39 ± 0.16 log CFU/coupon, respectively. When biofilms were considered mature (after 96 h), no significant difference was observed in the population recovered on Teflon and PVC as compared to the previous sampling point ($P > 0.05$). Conversely a significant increase in the number of attached cells was observed for stainless steel, nylon, and HDPE ($P < 0.05$), reaching 6.00 ± 0.08, 6.57 ± 0.20, and 7.11± 0.10 log CFU/coupon, respectively ($P < 0.05$).

Effect of single and simultaneous treatment application on the reduction of *L. monocytogenes*. Table 3.1 displays the individual and combined factors with significant effects on *L. monocytogenes* populations recovered for 24 h cell aggregates. Antimicrobial treatment, and the interaction of treatment and material, showed significant effects ($P < 0.05$). In contrast, application time or material alone did not show a significant impact against *L. monocytogenes* cells ($P > 0.05$). The effects of UV-C light alone, and of PAA 120 ppm, silver dihydrogen citrate, and lactic acid treatments alone and in combination on *L. monocytogenes* 24 h aggregates and 96 h mature biofilm populations, are shown in Table 3.2. UV-C light applied alone for 5 min was the least effective treatment, although its efficacy varied depending on the surface material. The lowest *L. monocytogenes* population reductions were observed on PVC, stainless steel, and Teflon, demonstrating a reduction of 2.01, 2.10, and 3.89 log CFU/coupon, respectively ($P < 0.05$). The highest log reduction caused by UV-C light was observed on nylon surfaces achieving a 6-log reduction/coupon, followed by HDPE. Overall, all the liquid sanitizers PAA (120 ppm), SDC, and lactic acid (4%) were effective against 24 h aggregates achieving levels below the limit of detection (~7.0 log reduction) regardless of the type of surface, when applied at both 30 s and 1 min duration. The simultaneous application of sanitizer and UV-C light for up to 1 min did not increase the effectiveness of the treatment ($P > 0.05$).

For mature biofilms, among the individual factors analyzed, both antimicrobial treatment and material had a significant impact against *L. monocytogenes* biofilms ($P < 0.05$; Table 3.1), while time did not show a significant effect ($P > 0.05$). The interaction between treatment and material was also significant ($P < 0.05$), indicating that the effectiveness of antimicrobial treatments varied depending on the type of surface material on which the biofilms were grown. Additionally, a significant three-way interaction was observed between material, treatment, and

time ($P < 0.05$), suggesting that the combined effects of these factors influenced microbial survival. The application of UV-C light alone resulted in significantly lower counts on stainless steel, nylon, and HDPE compared to controls ($P < 0.05$) while no significant changes were observed on PVC and Teflon compared to their controls ($P > 0.05$). Additionally, biofilm populations after 5 min of UV-C light treatment did not significantly differ from those treated for 1 min, regardless of the surface type ($P < 0.05$).

PAA and lactic acid were the most effective sanitizer treatments. When mature biofilms on stainless steel were exposed to PAA for 1 and 5 min, cell recovery was significantly lower as compared to untreated controls ($P < 0.05$), and no difference between the treatment time applications was observed ($P > 0.05$). Also, when nylon coupons were treated with PAA for 1 and 5 min no time effect was noticed ($P > 0.05$). A similar phenomenon was also reported for PVC, HDPE, and Teflon. When PAA treatments were simultaneously combined with UV-C for 1 min, no significant difference in recovered population was observed as compared to PAA alone on any material type (P = 0.847). However, extending the exposure time to 5 min resulted in a significant reduction of population as compared to 1 min application on HDPE ($P < 0.05$) while no difference was observed on stainless steel, nylon, PVC, and Teflon ($P > 0.05$).

The use of lactic acid alone for 1 and 5 min significantly ($P < 0.05$) reduced biofilm population on stainless steel, nylon, HDPE, and PVC compared to the controls. Treatment time did not affect cell recovery except for Teflon ($P > 0.05$). The simultaneous application of lactic acid and UV-C light did not significantly differ from lactic acid alone for biofilms grown on stainless steel, PVC, and HDPE, regardless of treatment time. On nylon, 5 min application of lactic acid and UV-C light resulted in lower cell counts compared to 1 min application of the same treatment ($P < 0.05$; note that this result comes from a single contrast, and not from the multiple

comparisons). A similar effect was observed for Teflon, where longer application time of lactic acid with UV-C light resulted in significantly lower recovered populations ($P < 0.05$).

The application of SDC alone for 1 min resulted in significantly lower recovered cell counts compared to controls on stainless steel, nylon, and HDPE ($P < 0.05$). The greatest efficacy was observed on inoculated nylon coupons, which yielded the lowest number of recovered biofilm cells. The simultaneous application of SDC and UV-C led to greater reductions on the nylon surface, while biofilms formed on PVC and Teflon seemed to be less affected by these treatments as compared to other surfaces.

Microscopy images. Surfaces of uninoculated materials showed different topographical characteristics (Figure 3.4 – uninoculated surfaces): HDPE and stainless-steel coupons were relatively uniform with no significant rough topography, nylon appeared to be more fibrous, Teflon had some irregularities in the texture, while PVC displayed fine cracks and shallow surface roughness. After 96 h the entire coupons were covered by microbial biomass (Figure 3.4 – 96 h mature biofilm). When mature biofilms were treated for 1 min with different sanitizers, biomass was visibly affected. In our images (Figure 3.4 – treatments), z-axis values were overall reduced following treatments, indicating a decrease in biofilm thickness and cell clustering. Sanitizer applications altered biofilm architecture across the different surfaces. SDC was effective in reducing biomass on nylon. PAA and lactic acid treatments modified biofilm structure and colonization also on stainless steel and HDPE coupons, changing cell distribution and clustering. Conversely, PVC and Teflon surfaces exhibited a higher z-axis value after treatments, reflecting greater biofilm thickness and suggesting that biofilm formed on these surfaces might be more difficult to eliminate as compared to biofilm formed on HDPE and stainless steel.

Discussion

The persistence of *L. monocytogenes* on hard-to-clean areas of processing equipment is largely attributed to the ability to aggregate, accumulate, and form biofilms, resulting in an increased risk of cross-contamination (Osek et al., 2022; Rohilla et al., 2024). In this research, we investigated the efficacy of commercially available sanitizers alone or simultaneously with UV-C light in controlling *L. monocytogenes* growth and accumulation for 24 h (biofilm-like aggregate) and 96 h (mature biofilms). For sanitation procedures, an automated chamber device was employed to ensure consistency across treatments and leverage a pressurized system application. Sanitizers applied for both 30 s and 1 min using a sprayer set at a flow rate of 400 ml/min effectively reduced 24 h aggregates of *L. monocytogenes* cells to levels below the limit of detection (~7 log reduction), regardless of the type of sanitizer and surface. The pressure generated by the sprayer system likely played a critical role in dislodging loosely attached cells through physical removal. Previous studies applying PAA or SDC via manual immersion sanitation methods reported lower *L. monocytogenes* reduction levels of early-stage biofilm cells (24 h) on similar materials such as nylon fabric (Stewart et al., 2025), and applying chlorine on stainless steel (Jiao et al., 2025). Conversely, in 96 h biofilms, the sprayer system did not cause *L. monocytogenes* population levels to fall below the detection limit, highlighting the importance of daily cleaning and sanitation events to prevent the formation of mature biofilms.

Previous research indicated that different strains of *L. monocytogene*s exhibit varying capacities for accumulation and biofilm production (Djordjevic et al., 2002). Strains that are moderate or strong biofilm producers may have a competitive advantage over weak producing strains, contributing to their persistence in a food processing environment (Di Ciccio et al., 2022). While our study assessed biofilm production based on biomass measurement, conflicting results

exist regarding the role of *L. monocytogenes* lineage on biofilm forming ability (Borucki et al., 2003; Di Bonaventura et al., 2008; Djordjevic et al., 2002; Kalmokoff & Austin, 2001). Di Bonaventura et al. (2008) did not find any relation between serotype and biofilm formation. Conversely, other researchers reported that serotypes 1/2a and 1/2b demonstrated increased ability to form biofilms compared to serotype 4b (Borucki et al., 2003; Keeney et al., 2018). Similarly, our results showed that *L. monocytogenes* 390-1 (serotype 1/2a) and *L. monocytogenes* 390-2 (serotype 1/2b) were moderate biofilm producers, whereas serotype 4b was classified as a weak biofilm producer.

Surface attachment and progressive biofilm formation by *L. monocytogenes* on surfaces plays a critical role in determining the efficiency of sanitation efforts. The biofilm stage of growth might play a role in influencing treatment efficacy as well, since early biofilms are more vulnerable to treatments (Klopper et al., 2023; Kloula Ben Ghorbal et al., 2022). In our research, we explored both early-stage biofilms (24 h accumulation) and mature biofilms (96 h aggregation).

All antimicrobial treatments were found to be less effective on PVC and Teflon surfaces compared to stainless steel, HDPE, and nylon. Similar findings were reported by Lake et al., (2024) where cleaning and disinfection using an alkaline cleaning agent and a peracetic acid-hydrogen peroxide based disinfectant (3%) on PVC surfaces resulted in lower reductions compared to stainless steel. PAA is widely used in the produce industry due to its broad antimicrobial activity. As an oxidizing agent, PAA releases hydroxyl radicals that disrupt biofilm matrix components (Chino et al., 2017; Lee et al., 2016). These reactive oxygen species damage cellular membranes and proteins, but because PAA also reacts with organic matter, including biofilm constituents, its activity within established biofilms may be slower and incomplete (Zhang et al., 2019). Hua & Zhu (2024) reported that treating 7-day old static *L. monocytogenes* biofilms on stainless steel,

Teflon, and rubber with PAA at 160 ppm achieved reductions of 3.7 – 4.3 log CFU/coupon. Stewart et al. (2025) achieved reductions of 4.45, 2.89, and 0.92 log CFU/coupon on HDPE, nylon, and wood surfaces, respectively, using 500 ppm PAA for 1 min on dynamically formed biofilms. In our experiments, lower reductions were observed across all surfaces (1.6 – 1.8 log CFU/coupon), likely due to the lower PAA concentration used (120 ppm) and differences in biofilm growth conditions. Tsagkari et al. (2022) noted that bacterial binding can be stronger under high shear conditions, such as those created by the CDC Biofilm Reactor used in our study, which may explain the lower reductions observed.

Silver ions are known to disrupt bacterial growth, primarily by interacting with thiol groups and nucleic acids, inhibiting cell division and damaging the cell envelope and intracellular contents (Jung et al., 2008). However, its effect within biofilms may be restricted, as Ivers et al. (2024) hypothesized that cells embedded deeper in the matrix are less metabolically active and therefore more tolerant. In this study, SDC treatment exhibited the highest activity against *L. monocytogenes* biofilms grown on nylon surfaces. Nevertheless, increased contact time did not result in greater reductions. Similarly, Manville et al. (2023b) reported that increasing SDC contact time from 2 min to 5 min did not significantly affect *L. monocytogenes* reduction on polycarbonate, nylon bag, and wood surfaces. Stewart et al. (2025) also observed no significant changes in *L. monocytogenes* biofilm populations recovered from HDPE, nylon, and wood surfaces when the application time was increased from 1 min to 2 min. Conversely, (Ivers et al., 2024) reported a significant effect of treatment time when SDC contact time was increased from 1 to 2 min on *Salmonella* biofilms grown on HDPE.

Lactic acid can enter the cell and alter the cytoplasmic membrane, resulting in leakage of proteins (Wang et al., 2015). Its activity against biofilms may also involve inhibition of cell

proliferation and interference with EPS production, which are critical for biofilm stability (Kostoglou et al., 2024). Furthermore, the low pH of lactic acid can enhance penetration, but its diffusion through biofilms may be hindered by ionic interactions with the EPS matrix, with the inhibition degree depending on biofilm's architecture and composition (Yang et al., 2009). In this study, lactic acid achieved the highest reduction levels against *L. monocytogenes* biofilms, with log reductions of 2.25, 2.09, and 1.80 log CFU/coupon on nylon, Teflon, and stainless steel, respectively. Similar reductions have been reported in previous studies using lactic acid at 4% on material surfaces such as stainless steel, and polycarbonate applied during 2 and 5 min (Manville et al., 2023b) or 10 min (Mendez et al., 2022). Studies evaluating lactic acid efficacy as a sanitizing agent at different concentrations on *L. monocytogenes* biofilms remains limited. Ban et al. (2012) reported less than 1 log *L. monocytogenes* biofilm reduction on stainless steel and PVC when using lactic acid at 2% for 30 s by using immersion application methods. For 24 h aggregates, lactic acid efficacy at 4% was comparable to that of 120 ppm PAA and SDC. Other studies have evaluated lactic acid at lower concentrations (0.1%) applied for up to 5 min showing limited inactivation on *L. monocytogenes* 24 h aggregates on stainless steel surfaces (Jee & Ha, 2023). Further research is warranted to assess effective concentrations of lactic acid as sanitizing agents, as no specific regulatory standards or guidelines using lactic acid have been established for sanitation of food contact surfaces.

The simultaneous application of UV-C light did not enhance reductions achieved by lactic acid or any other sanitizer evaluated. These observations suggest that longer UV-C exposure times might be necessary to observe synergistic antimicrobial effects. In our study, the shorter application times were selected based on industry practices. Bae & Lee (2012) applied longer exposure times of UV-C (3h) and reported significant reductions in *L. monocytogenes* cells on

stainless steel (2.08 log CFU/coupon). The same study found that 3 h of UV-C exposure was efficient in reducing *L. monocytogenes* cells on polypropylene. UV-C intensity also played a role in antimicrobial effects: in our experiments, UV-C intensity was measured within the range of 3 – 4.5 mW/cm^2, while previous studies reported much higher intensity levels. For example, Lippman et al. (2020) evaluated the combined use of UV-C light and PAA for lettuce decontamination and found that 30 mW/cm^2 UV-C combined with 80 ppm PAA for 2 min achieved a significantly higher log reduction of *Salmonella* as compared to PAA treatment alone. An increased reduction in *L. monocytogenes* biofilms was also reported by Kim et al. (2016) following UV-C and NaOCl treatment on stainless steel surfaces, where the largest synergistic effect was observed when NaOCl (200 ppm) was combined with UV-C at a dosage of 1800 mWs/cm^2.

To further explore the effect of sanitizers and UV-C light on *L. monocytogenes* biofilm architecture, we conducted microscopy analysis. Surface topography plays a critical role in bacterial attachment, subsequent aggregation, biofilm formation and cleanability of surfaces (Arnold & Bailey, 2000). Irregular features such as microscopic grooves and deep valleys, can hinder effective cleaning by providing sheltered areas for bacterial accumulation (Das et al., 2020; Hsu et al., 2013). Microorganisms often align themselves to valley and pillar structures to maximize the attachment area (Zheng et al., 2021). As a result, the cleanability of the surface largely depends on topographical features (Määttä et al., 2011). Previously, Waldhans et al. (2023) compared the cleanability of various conveyor belt materials, including thermoplastic polymers (polyurethane, polyoxymethylene, and polyolefins) and stainless steel, and found that thermoplastics generally exhibited lower cleanability. Tantratian et al. (2022) investigated *Vibrio parahaemolyticus* biofilms on stainless steel surfaces with different roughness levels and observed that biofilms adhered better to rougher stainless steel compared to smoother ones. Stainless steel

surfaces free of scratches and cuts were easier to clean and more effectively sanitized. Midelet & Carpentier (2002) tested *L. monocytogenes* attachment abilities on conveyor belt materials and reported that *L. monocytogenes* biofilms attached more strongly to polymers (PVC and polyurethane), as compared to stainless steel. Similar observations were made in our experiments, where higher levels of *L. monocytogenes* attachment were observed on PVC and Teflon surfaces. Similarly, Veluz et al. (2012) also observed higher *Salmonella* and *L. monocytogenes* attachment on plastic conveyor belt materials as compared to stainless steel (Veluz et al., 2012). Our observations, as well as the reported literature, support the concept that microbial early attachment allows cells to adhere more firmly to surfaces and demonstrate the importance of routine cleaning and sanitation procedures for effective microbial control.

Future research should prioritize assessing the effectiveness of automated sanitation procedures when organic matter and other residues are present, as these factors influence sanitizer efficacy. Moreover, due to the critical role of a cleaning step before sanitation, future studies should also explore efficient methods for removing biofilms prior to applying sanitizers. Lastly, this study was limited to flat surface material coupons, highlighting the need for additional investigations into non-hygienic surface designs (e.g., brushes, rollers, interlocking belting) under field conditions, as well as examining the long-term effects of these sanitizers on equipment wear.

Conclusion

This study highlights the significant influence of surface materials on biofilm architecture and surface cleanability. The type of surface material where biofilms were formed played a critical role in determining the efficacy of antimicrobial treatments, with different surfaces showing variation in biofilm formation and removal. The simultaneous application of UV-C light and chemical sanitizers, under conditions and parameters used in our studies, did not enhance

antimicrobial efficacy compared to using chemical sanitizers alone. Among the tested strategies, PAA at 120 ppm and lactic acid at 4% were found to be the most effective across all material types, achieving the highest reduction against *L. monocytogenes* biofilms. For 24 h aggregates, all treatments were significantly effective, with the exception of UV-C alone, regardless of application time and surface material type, highlighting the importance of using automated pressurized systems and daily cleaning and sanitation procedures to prevent *L. monocytogenes* biofilm formation on food processing surfaces.

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Tables

Table 3.1. Statistical analysis (ANOVA) of the different parameters evaluated during treatments of *L. monocytogenes* aggregates and mature biofilms grown on different surfaces.

Parameters	P-value Aggregates	P-value Mature biofilms
Treatment	<0.05a	<0.05a
Time	0.066	0.47
Treatment x time	0.58	<0.05a
Material	0.81	<0.05a
Treatment x material	<0.05a	<0.05a
Time x material	0.915	0.995
Treatment x time x material	0.992	<0.05a

[a] Values marked with lowercase letters denote statistical significance with $P < 0.05$.

Table 3.2. Recovered *L. monocytogenes* aggregates (24 h) and mature biofilms grown on different materials used in this study after exposure to sanitizers alone or simultaneously with UV-light for 1- and 5-min exposure (log CFU/coupon).

Material	Intervention	*L. monocytogenes* aggregates		*L. monocytogenes* mature biofilms	
		log CFU/coupon (30 s exposure)	log CFU/coupon (1-min exposure)	log CFU/coupon (1-min exposure)	log CFU/coupon (5-min exposure)
Stainless Steel	Control	8.90 ± 0.22a		6.72 ± 0.37a	
	UV	(5min) 6.88 ± 0.26b		(5min) 6.19 ± 0.24ab	
	PAA	2.44 ± 0.00c	2.44 ± 0.00c	5.12 ± 0.15d	4.90 ± 0.2d
	PAA + UV	2.44 ± 0.00c	2.44 ± 0.00c	5.13 ± 0.11cd	4.84 ± 0.23d
	SDC	2.58 ± 0.31c	2.44 ± 0.00c	5.32 ± 0.07bcd	4.99 ± 0.11d
	SDC + UV	2.72 ± 0.51c	2.76 ± 0.71c	4.88 ± 0.37d	4.29 ± 0.22d

	Lactic acid	2.44 ± 0.00c	2.44 ± 0.00c	4.92 ± 0.49d	4.99 ± 0.22d
	Lactic acid + UV	2.44 ± 0.00c	2.44 ± 0.00c	5.33 ± 0.04bcd	4.95 ± 0.24d
Nylon	Control	9.00 ± 0.10a		7.06 ± 0.42a	
	UV	(5 min) 3.07 ± 1.46b		(5 min) 6.43 ± 0.25b	
	PAA	2.44 ± 0.00b	2.44 ± 0.00b	5.25 ± 0.36de	5.15 ± 0.56def
	PAA + UV	2.44 ± 0.00b	2.44 ± 0.00b	5.56 ± 0.24bcd	4.94 ± 0.63def
	SDC	2.83 ± 0.68b	2.59 ± 0.25b	3.55 ± 0.33g	4.03 ± 0.85fg
	SDC + UV	3.01 ± 0.81b	2.96 ± 1.09b	3.55 ± 0.45g	4.18 ± 1.26efg
	Lactic acid	2.44 ± 0.00b	2.44 ± 0.00b	4.81 ± 0.45def	5.28 ± 0.34cde
	Lactic acid + UV	2.44 ± 0.00b	2.44 ± 0.00b	5.41 ± 0.37cdA	4.47 ± 0.41defgA
PVC	Control	9.22 ± 0.24a		8.67 ± 0.31a	
	UV	(5 min) 7.21 ± 0.29b		(5 min) 8.48 ± 0.16ab	
	PAA	2.44 ± 0.00c	2.44 ± 0.00c	7.45 ± 0.36cd	7.53 ± 0.31cd
	PAA + UV	2.44 ± 0.00c	2.44 ± 0.00c	7.61 ± 0.3bcd	7.81 ± 0.3bcd
	SDC	2.88 ± 0.64c	2.65 ± 0.46c	8.05 ± 0.1abcd	7.78 ± 0.27bcd
	SDC + UV	3.39 ± 1.44cA	2.58 ± 0.19cA	7.93 ± 0.3abcd	7.81 ± 0.22bcd
	Lactic acid	2.44 ± 0.00c	2.44 ± 0.00c	7.56 ± 0.17cd	7.75 ± 0.23bcd
	Lactic acid + UV	2.44 ± 0.00c	2.44 ± 0.00c	7.27 ± 0.25d	7.15 ± 0.39d
HDPE	Control	9.06 ± 0.03a		7.15 ± 0.41a	
	UV	(5 min) 3.75 ± 0.92b		(5 min) 6.14 ± 0.39b	
	PAA	2.74 ± 0.66c	2.44 ± 0.00c	5.61 ± 0.11bcd	5.02 ± 0.28de
	PAA + UV	2.44 ± 0.00c	2.44 ± 0.00c	5.42 ± 0.14bcdA	4.64 ± 0.51deA
	SDC	2.95 ± 0.81bc	2.44 ± 0.00c	5.06 ± 0.49de	5.08 ± 0.46cde
	SDC + UV	2.78 ± 0.76c	2.44 ± 0.00c	4.17 ± 0.25e	4.65 ± 0.17de

	Lactic acid	2.99 ± 0.74bc	2.44 ± 0.00c	5.62 ±0.34bcd	5.25 ± 0.29bcde
	Lactic acid + UV	2.44 ± 0.00c	2.44 ± 0.00c	5.63 ± 0.13bcd	5.07 ± 0.38cde
Teflon	Control	8.96 ± 0.15a		8.76 ± 0.34a	
	UV	(5 min) 5.07 ± 1.33b		(5 min) 8.64 ± 0.12ab	
	PAA	2.44 ± 0.00c	2.44 ± 0.00c	7.20 ± 0.39cdefg	7.14 ± 0.09cdefg
	PAA + UV	2.44 ± 0.00c	2.44 ± 0.00c	7.08 ± 0.09defg	6.85 ± 0.17efg
	SDC	2.74 ± 0.66c	2.44 ± 0.00c	8.03 ± 0.18abcde	8.26 ± 0.18abc
	SDC + UV	2.97 ± 0.64c	2.44 ± 0.00c	8.13 ± 0.18abcd	7.78 ± 0.05bcdef
	Lactic acid	2.44 ± 0.00c	2.44 ± 0.00c	7.48 ± 0.63cdefgA	6.67 ± 0.56fgA
	Lactic acid + UV	2.44 ± 0.00c	2.44 ± 0.00c	7.42 ± 0.2cdefgA	6.57 ± 0.19gA

[a] Results are presented as means ± standard deviation.

[b] A value with 0.00 standard deviation indicates that all replicates were at or below the limit of detection (2.44 log CFU/coupon) for 24 h aggregates.

[c] Lowercase letters compare treatments within the same material towards the control and among the different sanitizer treatments (including different times of exposure). If two cells within a material share a letter, no significant differences were found between those treatments.

[d] Capital letters indicate that there is a significant difference ($P < 0.05$) between the two exposure times for the same treatment and material (i.e., row-wise comparison). Note that the lowercase letters and capital letters may convey different messages (e.g., two elements contain the same lowercase letter but have a capital letter). This phenomenon can be explained due to a more conservative approach in Tukey-adjusted multiple comparisons, that needs more statistical evidence (i.e., lower standard errors or greater mean differences) to detect significant differences. Tukey-adjusted multiple comparisons avoid global type I error inflation (i.e., erroneously rejecting at least one null hypothesis when that hypothesis was false) that typically happens with many pairwise comparisons are performed simultaneously, essentially by increasing the alpha (probability of committing type I error) for each pairwise comparison greater than 0.05, for a global alpha of 0.05.

Figures

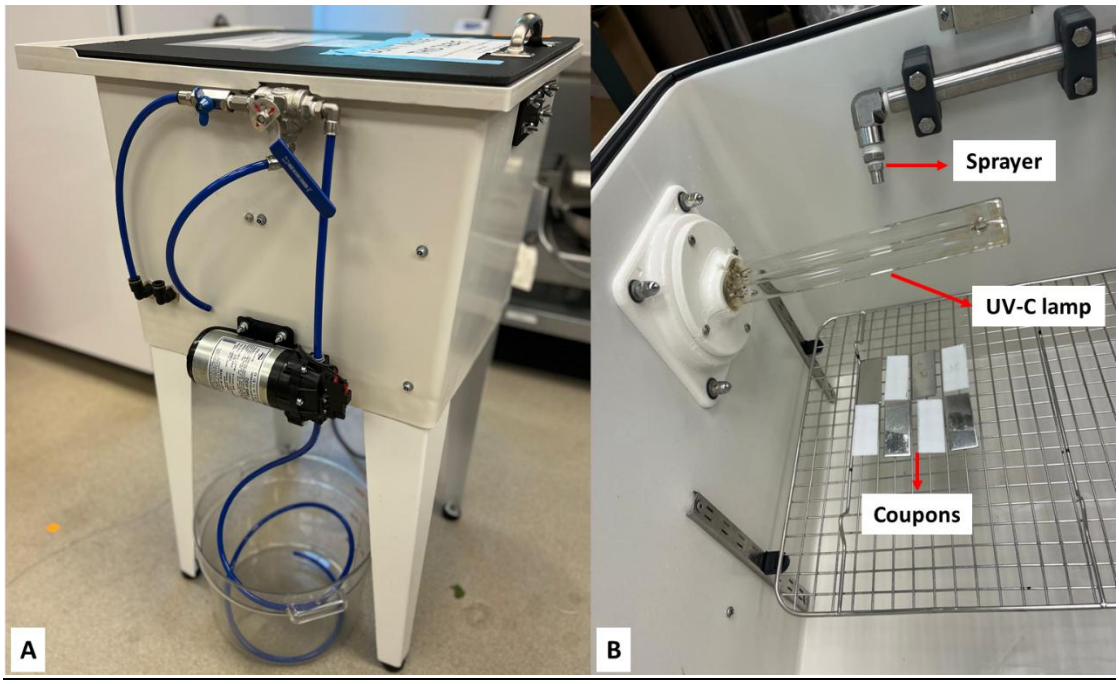


Figure 3.1. (A) Laboratory scale device equipped with a UV-C lamp and sanitizer spray system (B) Location of coupons for treatments inside the device for 24 h aggregates.

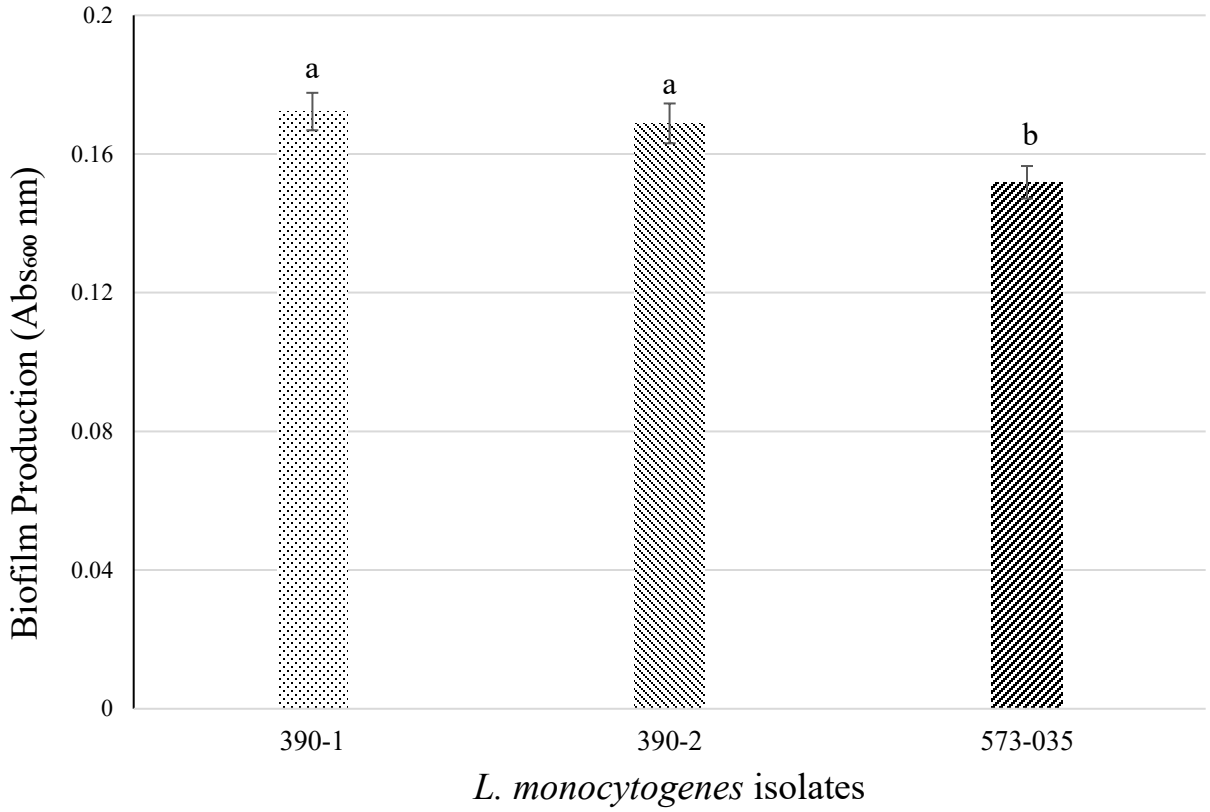


Figure 3.2. Biofilm production by *L. monocytogenes* strains used in this study, measured by crystal violet staining. Average absorbance (600 nm) is represented by column and standard error by bars. Small letters indicate statistical differences in biofilm production among the strains.

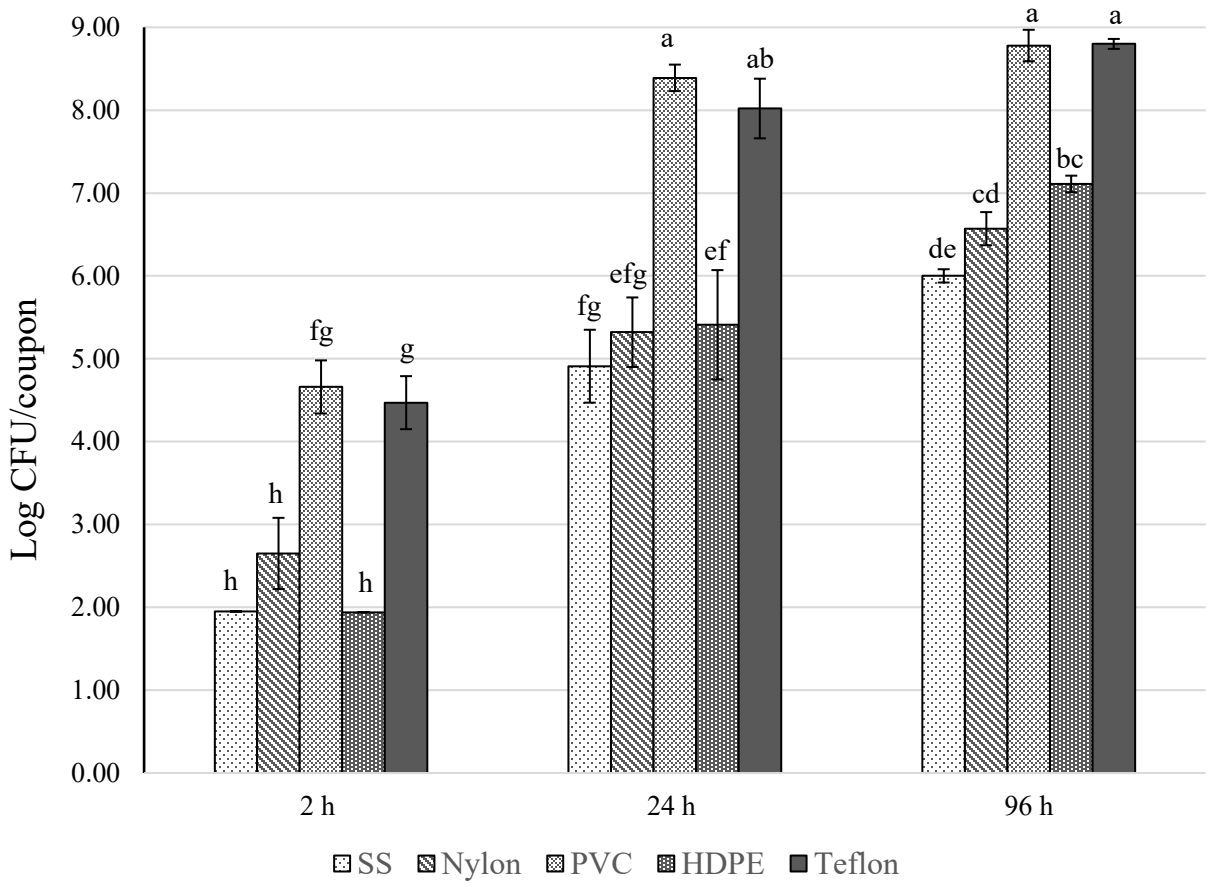


Figure 3.3. *L. monocytogenes* (Log CFU/coupon) remaining populations on stainless steel, nylon, PVC, HDPE, and Teflon after 2, 24, and 96 h incubation and rinse step. Small letters indicate statistical differences between incubation times.

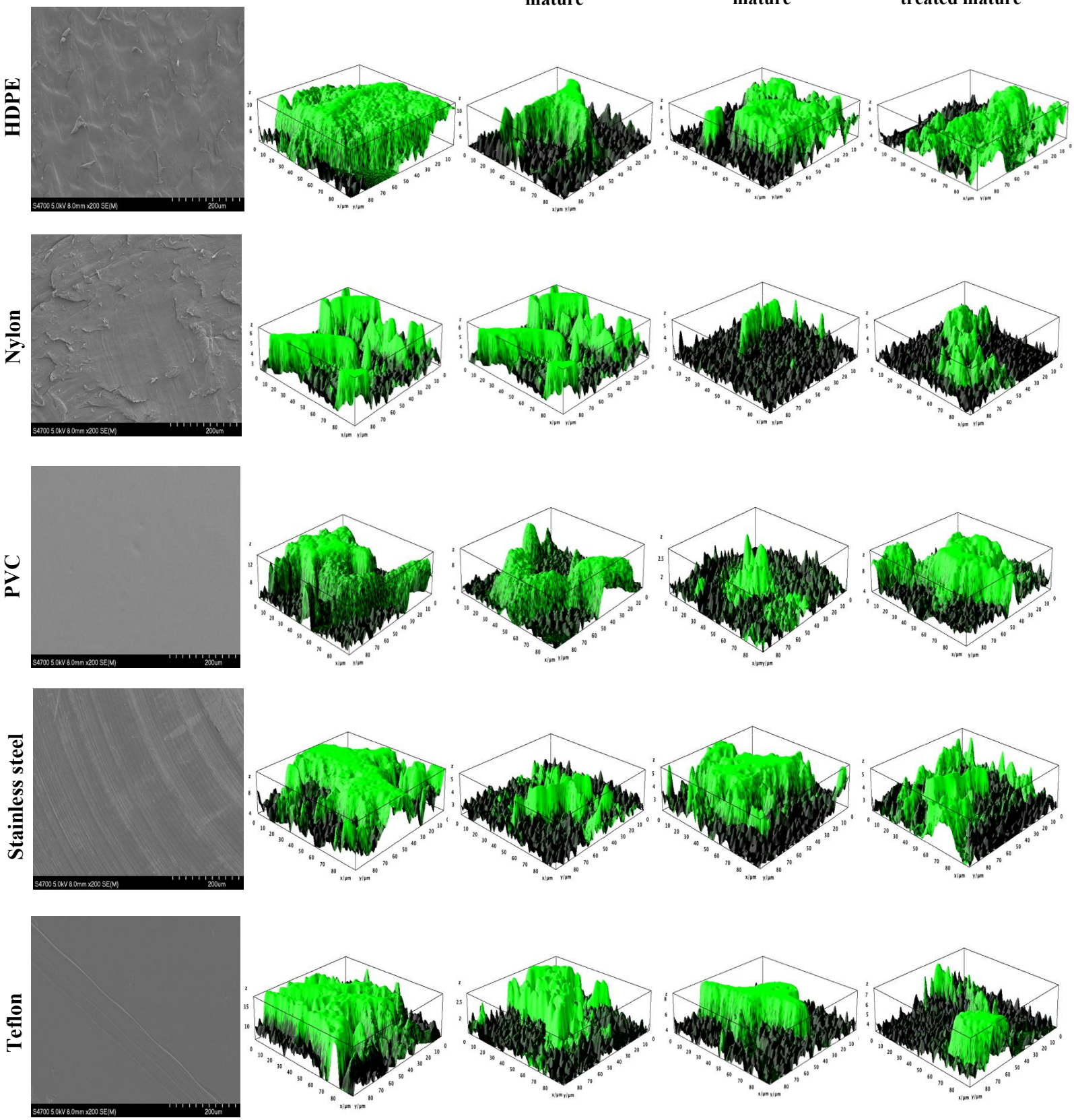

Uninoculated surfaces
96-h mature biofilm
PAA treated mature
SDC treated mature
Lactic acid treated mature
HDPE
Nylon
PVC
Stainless steel
Teflon
S4700 5.0kV 8.0mm x200 SE(M)
200um

Figure 3.4. SEM images of uninoculated clean surfaces and 3D surface projections of *L. monocytogenes* biofilms on HDPE, nylon, PVC, stainless steel, and Teflon after 96-h of growth in CDC Biofilm Reactor, followed by 1 min treatments with PAA, SDC, and lactic acid. Biofilm samples were analyzed by Laser Scanning Confocal Microscopy (LSCM), and all 3D images were constructed using ImageJ2. Scale bars for SEM images represent 200 μm.

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Chapter 4 - Transcriptomic Response of *L. monocytogenes* biofilms to commercially available sanitizers

Abstract

Listeria monocytogenes is a persistent foodborne pathogen capable of surviving routine sanitation procedures in food processing environments. Exposure to environmental stress can induce cross-protection against subsequent challenges, as observed under oxidative, acidic, and low-temperature conditions, commonly used as antimicrobial interventions in the food industry. Biofilm-associated cells further enhance persistence and tolerance, complicating sanitation efforts. In this study, biofilms of a multi-strain *L. monocytogenes* cocktail were developed at 25 ± 2 °C for 48 h and exposed for 15 s to sublethal sanitizer concentrations of lactic acid (0.4% (v/v)), chlorine (10 ppm), peracetic acid (PAA, 10 ppm), and silver dihydrogen citrate (SDC, 5%), with untreated biofilms as controls. RNA sequencing revealed that oxidative sanitizers, particularly chlorine and PAA, elicited the most extensive transcriptional reprogramming, resulting in 128 and 64 differentially expressed genes (DEGs), respectively, whereas lactic acid and SDC caused minimal transcriptional changes. GO and KEGG enrichment analyses indicated that chlorine exposure upregulated genes involved transcriptional regulation, the phosphotransferase system (PTS), and stress-responsive signaling, while PAA enriched pathways associated with phosphate ion transport and ABC transporter. These findings demonstrate that sublethal sanitizer exposure triggers coordinated transcriptional responses encompassing stress defense, metabolic adaptation, and transport regulation, with sanitizer type and chemistry influencing the magnitude and specificity of gene expression responses.

Introduction

Listeria monocytogenes is a virulent foodborne pathogen capable of persisting in food processing environments, thereby increasing the risk of cross-contamination of food products (Casey et al., 2014a; Osek et al., 2022). Its ability to attach to surfaces and form biofilms represents a major concern for the food industry (Nguyen Trang et al., 2023; Poimenidou et al., 2009). When in biofilms, *L. monocytogenes* exhibits enhanced resistance to environmental stressors, making it more difficult to eliminate and reducing the efficacy of cleaning and sanitation procedures (Mazaheri et al., 2021). Bacterial metabolic processes are the primary targets of disinfection treatments, and exposure to sanitizers can prompt *L. monocytogenes* to alter metabolic pathways, including respiration energy production, carbohydrate utilization, lipid, nucleic acid, and amino acid metabolism (Rajalingam & Van Haute, 2025).

Antimicrobial agents are generally more effective against planktonic cells than biofilm. Bacterial adaptive responses and biofilm-associated protective mechanisms play a critical role in this reduced susceptibility (Kim et al., 2008). Moreover, variation in sanitizer efficacy due to factors such as the presence of organic matter, inaccurate concentration and exposure time, surface characteristics, or equipment design might limit cleaning and sanitation efficacy resulting in *L. monocytogenes* being exposed to sublethal concentrations of antimicrobials (Bansal et al., 2018). Repeated or prolonged exposure to sublethal stress can further enhance bacterial adaptation (Bansal et al., 2018). It has been reported that the persistence of certain strains of *L. monocytogenes* in food processing environments was associated with repeated and prolonged sublethal sanitizer exposure (Martínez-Suárez et al., 2016).

L. monocytogenes possesses well-characterized stress response regulators (Arvaniti et al., 2025; Pleitner et al., 2014). The general stress response in *L. monocytogenes* is primarily regulated

by the alternative sigma factor σ^B, which associates with RNA polymerase and directs it to σ^B dependent promoters, resulting in transcriptional reprogramming (NicAogáin & O'Byrne, 2016). This enables the expression of protective functions: The σ^B regulon, also referred to as General Stress Response (GSR), positively regulates approximately 300 genes involved in stress adaptation and virulence of *L. monocytogenes* (Arvaniti et al., 2025; NicAogáin & O'Byrne, 2016; Pleitner et al., 2014). This regulon has been shown to play a key role in both planktonic and biofilm resistance to sanitizers such as benzalkonium chloride and peracetic acid during short term exposure (NicAogáin & O'Byrne, 2016).

Peracetic acid (PAA) is an organic peroxy acid that is widely used in the food industry for the sanitation of food-contact surfaces (Ao et al., 2021; Leggett et al., 2016). PAA exerts its antimicrobial activity through oxidation and the generation of reactive oxygen species (ROS) (Arvaniti et al., 2025). Bacteria have evolved multiple defense mechanisms to protect against ROS-mediated oxidative stress, including the production of scavenging enzymes (Arvaniti et al., 2025; Cesinger et al., 2021). According to Cesinger et al. (2021) in *L. monocytogenes*, the peroxidase genes *cdhC* and *kat* are required for aerobic growth in vitro, whereas *fri* and *ahpA* are essential for survival under acute peroxidase stress. Also, OhrA-OhrR system is highly efficient antioxidant system that plays role as a response to organic peroxidases (Arvaniti et al., 2025). Chlorine is another strong oxidizing agent and one of the most widely used sanitizers in the food industry for surface decontamination (Kim et al., 2008; Osek et al., 2022). This chemical can inactivate cells through multiple mechanisms, including oxidation of sulfhydryl enzymes and amino acids, inhibition of protein synthesis, and disruption of membrane integrity (CDC, 2023). Pleitner et al. (2014) reported that a significant number of genes were differentially expressed in *L. monocytogenes* following sublethal exposure to oxidative stress, including genes involved in

heat shock response and redox reactions. In addition, genes associated with the electron transport chain, NAD^+ regeneration, and ATP synthesis were differentially regulated under antimicrobial treatment (Rajalingam & Van Haute, 2025).

Lactic acid is another antimicrobial used in the food industry, with a broad efficacy spectrum (Boomsma et al., 2015.; Byun et al., 2021). The antimicrobial activity of lactic acid is largely attributed to its undissociated form of the acid to diffuse across the cell membrane (Alakomi et al., 2000). Once inside, the acid dissociates into hydrogen cations and acid anions. The accumulation of these ions disrupts the proton motive force, lowers intracellular pH and interferes with metabolism (Alakomi et al., 2000; Stasiewicz et al., 2011). Lactic acid stress has been reported to induce the upregulation of numerous stress-responsive genes in *L. monocytogenes*, including σ^B regulon (Cortes et al., 2020).

Silver ions are well recognized for their broad-spectrum antimicrobial activity and have been applied across various industries, including food processing (Jo et al., 2007). Their antimicrobial action is primarily associated with interactions between silver ions and thiol groups of cellular proteins, leading to enzyme inactivation (Jo et al., 2007; Jung et al., 2008). In addition, silver ions can interact with DNA, disrupting replication through enhanced pyrimidine dimerization (Jo et al., 2007). The antimicrobial efficacy and stability of silver are further enhanced when combined with citric acid to form silver dihydrogen citrate (SDC), a stabilized complex that facilitates the controlled release of silver ions (Buckley et al., 2018; Manuel et al., 2017). Bacteria can internalize SDC by recognizing citric acid as a potential nutrient source, allowing silver ions to enter the cytoplasm, where they interfere with DNA synthesis and inactivate essential proteins, ultimately leading to cell death (PURE Bioscience Inc., 2016).

Understanding the underlying mechanisms that enable *L. monocytogenes* biofilms to respond and adapt under sanitizer exposure is essential for improving control strategies in food processing environments. Transcriptomic analysis offers a powerful tool to characterize gene expression changes associated with environmental stress responses.

Therefore, this study aimed to investigate the transcriptomic responses of *L. monocytogenes* biofilms following sublethal exposure to 10 ppm PAA, 10 ppm chlorine, 0.4% lactic acid, and 5% SDC. The objective was to elucidate the stress response mechanisms that may contribute to the adaptation and survival of *L. monocytogenes* under sanitizer stress in food processing environments.

Materials and Methods

Bacterial strains. Three *L. monocytogenes* strains were selected for this study: 390-2 (serotype 1/2b), 390-6 (serotype 1/2a), both environmental isolates recovered from the 2011 cantaloupe outbreak (CDC, 2012); and 573-035 (serotype 4b), a clinical isolate obtained from the 2014-2015 caramel apple outbreak (CDC, 2015). The strains were whole-genome sequenced at the U.S. Food and Drug Administration – Human Foods Program (HFP). All strains were preserved at -80 °C in CryoCare Bacteria Preserver beads (Key Scientific, Stamford, TX, USA).

Genomic DNA extraction. Frozen cultures were streaked on Tryptic Soy Agar (TSA; BD Difco, USA) and incubated at 37 ± 2 °C for 24 h. Single colonies were inoculated into 10 mL Tryptic Soy Broth (TSB) and incubated at 37 ± 2 °C for 18-20 h with agitation (150 rpm). Genomic DNA from each strain was extracted using the Monarch HMW DNA Extraction kit (New England Biolabs, Ipswich, MA, USA) according to the manufacturer's instruction. DNA concentration was determined by the Qubit dsDNA BR Assay kit on a Qubit 4 Fluorometer (Invitrogen, Carlsbad,

CA, USA) according to the manufacturer's instructions. The DNA integrity was determined using a Femto Pulse System (Agilent, Santa Clara, CA, USA).

Whole-genome sequencing and assembly using long reads. DNA extracted from each strain was sequenced using the GridION sequencing Device Mk1 (Oxford Nanopore Technologies, Oxford, UK). The sequencing libraries were prepared during Ligation Sequencing Kit v14 (SQK-LSK114) with Native Barcoding Kit (EXP-NBD114). Libraries were loaded onto FLO-MIN114 (R10.4.1) flow cells, according to the manufacturer's instructions, for 48 hours (Oxford Nanopore Technologies). Flow-cell QC and mux scans were performed in MinKNOW software version 22.12.5 using the super-accurate basecalling model (SUP, basecall_model—dna_r10.4.1_e8.2_260bps_sup@v3.5.2). The genomes for each strain were obtained by de novo assembly using the Flye program v2.9.2 (Kolmogorov et al., 2019), using the following parameters: --nano-hq, --read-error 0.03, and -i 4.

In parallel, 100 ng of DNA per strain was used to prepare Illumina libraries using the Illumina® DNA Prep (M) Tagmentation (Illumina, San Diego, CA, USA), according to the manufacturer's instructions. Libraries were dual-indexed, quantified by Qubit and checked on a TapeStation D1000. Pooled libraries were sequenced on an Illumina MiSeq platform with the MiSeq V3 kit using 2 × 250 bp paired-end chemistry (Illumina, San Diego, CA, USA), according to the manufacturer's instructions, at 100X coverage. Reads were trimmed with Trimmomatic v0.36 using default parameters.

The complete genome for each strain was obtained by a de novo hybrid assembly using both nanopore and MiSeq data for that strain with Unicycler v0.5.0 (Wick et al., 2017). Unicycler assembled the chromosome and plasmids as circular closed and oriented the chromosome to start at the *dnaA* gene.

Biofilm growth. Frozen cultures were streaked onto Tryptic Soy Agar (TSA; BD Difco, Sparks, MD, USA) and incubated at 37 ± 2 °C for 24 h. A single colony from each plate was transferred into fresh 10 ml of Tryptic Soy Broth (TSB; BD Difco, Sparks, MD, USA) and incubated at 37 ± 2 °C for 24 h with agitation at 70 rpm. To prepare a multi-strain cocktail, one ml of each culture was combined in a 1:1:1 ratio. The initial bacterial concentration of the cocktail was determined by serial dilution 10-fold dilution and plating on TSA.

Biofilm formation was performed using a modified version of the protocol described by Dunn et al. (2020). Briefly, 3 ml of fresh TSB (20 g/l) was dispensed into sterile 50-mm petri dishes, followed by inoculation with 300 µl of the *L. monocytogenes* multi-strain cocktail. Petri dishes were incubated statically at 25 ± 2 °C for 48 h to allow biofilm development.

Sublethal concentrations and sanitizer application. Peracetic acid (PAA) (SaniDate 15.0, BioSafe Systems, LLC, East Hartford, CT), chlorine (Diversey, Fort Mill, SC), lactic acid (Purac®, Corbion, Blair, NE, USA), and silver dihydrogen citrate (SDC) containing 4.85% citric acid and 0.003% silver ions (Pure Bioscience, El Cajon, CA) were the sanitizers studied in this research. Sublethal conditions for each sanitizer were determined as the combination of concentration and exposure time that resulted in at least 90% recovery of the biofilm population compared to untreated controls (Arvaniti et al., 2025). Preliminary experiments (data not shown) were conducted to identify appropriate conditions, and working solutions of 10 ppm PAA, 10 ppm chlorine, 0.4% v/v lactic acid were selected for subsequent experiments and prepared according to manufacturer's instructions. The concentrations of the prepared sanitizers were verified prior to experiments using PAA, lactic acid, and chlorine titration kits (Chemworld, Kennesaw, GA). SDC was used as supplied without further dilution. All sanitizer treatments were applied for 15 s and untreated biofilms served as controls.

Following sanitizer preparation, biofilms were treated with the selected sanitizers. Spent TSB was first removed from the petri dishes by pipetting without disturbing the biofilm layer. Then, 1 mL of sanitizer was carefully pipetted onto the biofilm surface to ensure uniform coverage while avoiding disruption of the cell monolayer. After 15 s exposure, the sanitizer was removed by pipetting, and 1 ml of neutralizing broth was added. Single-strength D/E Neutralizing Broth () was used to neutralize SDC and lactic acid, whereas double-strength D/E was used for chlorine and PAA treatments. The neutralizer was removed after 15 s, and 1 ml of ice-cold 15% ethanol was immediately added to resuspend the biofilm cells. The resulting suspension was transferred into tubes containing 9 ml of ice-cold 15% ethanol. Serial 10-fold dilutions were prepared in 0.1% peptone water (Bacto, Sparks, MD, USA), followed by spread plating on TSA. The remaining ethanol-cell suspension was centrifuged at 4 °C for 12 min at 4,300 x g. The supernatant was aspirated, and the pellet was resuspended in 1 ml of ice-cold 15% ethanol. Cells were harvested by centrifugation at 14,000 x g for 1 min. The resulting pellet was resuspended in 100 µl of lysozyme-containing molecular-grade 1 X Tris-EDTA buffer (3 mg/ml), vortexed, and incubated at 22 ± 2 °C for 10 min to achieve cell lysis.

RNA extraction and sequencing. Following cell lysis, total RNA was extracted using Norgen RNA Total Plus Kit (Norgen Biotek, Thorold, ON, Canada) according to the manufacturer's instructions. Extracted total RNA concentration was measured using the Qubit™ RNA High Sensitivity Assay on a Qubit 4 Fluoremeter (Thermo Fisher Scientific, Waltham, MA, USA), and RNA integrity was assessed with an Agilent 4150 TapeStation system (Agilent Technologies, Santa Clara, CA, USA) with the High Sensitivity RNA ScreenTape Assay (Agilent, Germany).

cDNA flibraries were prepared using the Illumina Stranded Total RNA Prep, Ligation with Ribo-Zero Plus kit (Illumina, San Diego, CA, USA) following the manufacturer's protocol. The concentrations of each dual-indexed amplified library were determined using the Qubit DNA High Sensitivity Assay kit, and library fragment size distribution was evaluated with the Agilent 2200 TapeStation system and D1000 ScreenTape Assay (Agilent, Germany). Libraries were then normalized, and equal volumes of (10 µl) of each library were pooled and loaded onto a P3 flow cell. Sequencing was performed on an Illumina NextSeq 2000 platform using a 300-cycle kit to generate pair-end reads.

RNAseq data analysis and statistical analysis. The reference genome was constructed using the pangenome derived from the three *L. monocytogenes* strains. Each individual genome was first annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al., 2016). The pangenome, defined at 90% protein identity, was then determined for the three strains using Roary (v3.12.0). A transcriptome index for Salmon was subsequently built using the pangenome FASTA output generated by Roary. Pseudo-alignment of the raw reads was performed against this transcriptome index, and transcript abundances were quantified using Salmon (v1.10.2). Differential gene expression analysis of sanitizer-treated and untreated biofilms was performed using the *DESeq2* package (v1.46.0) in R (v4.4.2). Differentially expressed genes (DEGs) with a |log2FoldChange| ≥1 and an adjusted P value < 0.05 were reported. The Database for Annotation, Visualization and Integrated Discovery (DAVID) (DAVID Functional Annotation Bioinformatics Microarray Analysis) was used for gene enrichment analysis of the annotated DEGs with ease score set to 0.05, and padj <0.05 (https://davidbioinformatics.nih.gov/tools.jsp). R package clusterProfiler v. 4.14.6 was used to compare and visualize DAVID derived enriched

gene ontology (GO) terms and KEGG pathways with a p-value cut off of 0.05 and Bonferroni correction.

Statistical analysis. All experiments were performed with four independent biological replicates. Variation in *L. monocytogenes* biofilm populations across sanitizer treatments was evaluated by one-way analysis of variance (ANOVA). Where significant differences were observed, Tukey's honestly significant difference (HSD) test was used for post hoc multiple comparisons. Statistical significance was set at $P < 0.05$.

Results

Survival of *L. monocytogenes* biofilms following sublethal sanitizer exposure. The recovered populations of *L. monocytogenes* biofilms following sublethal sanitizer treatments are shown in Figure 4.1. Compared to untreated controls, treatments with 10 ppm chlorine, 10 ppm peracetic acid (PAA), or 0.4% lactic acid for 15 s resulted in approximately a 10% reduction in viable biofilm population. Similarly, exposure to 5% silver dihydrogen citrate (SDC) for 15 s yielded viable counts of approximately 7 log CFU/cm^2, indicating that the applied sanitizer concentrations with the contact time were sublethal and permitted substantial population recovery.

Effect of sanitizer on global transcriptomic shifts. Principal component analysis (PCA) of the global transcriptomic profiles was conducted to examine the overall variation among treatments. The PCA plot (Figure 4.2) showed the first two principal components accounted for 26.9% and 24.9% of the total variance, respectively. Distinct clustering patterns were observed between control and treated samples, confirming sanitizer-induced alterations in transcriptional activity. Biofilms exposed to chlorine and PAA showed the greatest separation from the control group along the principal components, reflecting a more pronounced effect on global transcriptional regulation under oxidative stress. In contrast, biofilms treated with lactic acid and

SDC clustered closely with the control, suggesting comparatively limited effects on overall gene expression.

Differential gene expression and overlap among treatments. To further characterize the transcriptomic response of *L. monocytogenes* biofilms to sanitizer stress, differentially expressed genes (DEGs) were identified for each treatment relative to the untreated control. The UpSet plot (Figure 4.3) illustrates the number of unique and shared DEGs among the different sanitizers. Each sanitizer induced a distinct transcriptional response, with chlorine and PAA generating the highest number of unique DEGs, suggesting that oxidative sanitizers elicit more specific stress responses. In contrast, lactic acid and SDC triggered comparatively few transcriptional changes. Chlorine exposure resulted in the most extensive transcriptional response, with 128 total DEGs, including 107 upregulated and 21 downregulated genes. Of these, 78 DEGs were unique to chlorine, indicating a highly specific oxidative stress response. Notably, 25 upregulated genes were shared between chlorine and PAA. PAA exposure also resulted in a moderate transcriptional response, with 64 total DEGs. Of these, 33 upregulated and 2 downregulated genes were specific to this treatment, indicating both shared and sanitizer-specific regulatory effects. Conversely, lactic acid exposure resulted in only a single DEG, which overlapped with both chlorine and PAA, whereas SDC affected five upregulated genes, two of which were shared with PAA.

Overall, oxidative sanitizers (chlorine and PAA) triggered the most pronounced transcriptional reprogramming, whereas lactic acid and SDC had minimal impact on gene transcriptional changes.

Enriched pathways and Gene Ontology. To characterize functional changes in response to sanitizer exposure, DEGs were separated into upregulated and downregulated groups based on $\log_2$FoldChange. Each set was analyzed independently for enriched Gene Ontology (GO) terms

and KEGG pathways associated with chlorine or PAA treatment. Figure 4.4A and 4.4B present the enriched GO terms and KEGG pathways associated with chlorine treatment. GO enrichment of chlorine-upregulated genes revealed transcriptional regulatory functions, indicating that chlorine exposure triggers regulatory reprogramming. Significantly (adjusted p-value < *0.05*) enriched GO terms included DNA-binding transcription factor activity, DNA binding, and regulation of DNA-templated transcription, suggesting activation of global transcriptional control mechanism. In addition, enrichment of the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) indicated potential shifts in carbohydrate transport and metabolic adaptation (Casey et al., 2014b). The enrichment of terms such as phosphatase activity and negative regulation of DNA-templated transcription suggests activation of stress-responsive signaling and regulatory control mechanisms (Sajid et al., 2015). KEGG pathway analysis (Figure 4.4B) supported these findings, with the phosphotransferase system (PTS) pathway significantly enriched, suggesting a coordinated response involving environmental sensing, metabolic adjustment, and membrane transport under chlorine-induced stress (Rajalingam & Van Haute, 2025).

For PAA, the only significantly enriched GO term was phosphate ion transmembrane transport **(**adjusted p-value < *0.05***)**, indicating possible alterations in ion homeostasis during oxidative stress (Figure 4.5A). In contrast, KEGG pathway analysis revealed enrichment of ABC transporters (Figure 4.5B), implying enhanced efflux or nutrient uptake mechanisms in response to sanitizer exposure (Joseph et al., 2006). The two-component system pathway was also enriched, suggesting activation of sensory and regulatory stress response mechanisms under PAA treatment.

Overall, these results indicated that oxidative sanitizers, particularly chlorine and PAA, induced both global regulatory reprogramming and metabolic adaptation in *L. monocytogenes* biofilms, facilitating survival under sublethal sanitizer stress conditions.

Discussion

The mode of action of sanitizers varies depending on their chemical composition and the cellular response of the target pathogen (Rajalingam & Van Haute, 2025). Sanitizers generally exert their antimicrobial effects by disrupting the cell membrane, causing leakage of intracellular components, damaging nucleic acids, and/or inducing oxidative stress through the generation of reactive oxygen species (ROS). The resulting imbalance in redox homeostasis can inhibit antioxidant enzyme activity and impair essential cellular functions. Bacterial stress adaptation and tolerance to sanitizer exposure may arise from changes in the expression of regulatory genes and stress response pathways (Schulz et al., 2023).

Oxidative stress has been shown to cause transcriptional reprogramming in *L. monocytogenes* (Pleitner et al., 2014). In a study by Pleitner et. al (2014), exposure of *L. monocytogenes* to 300 mg/l chlorine dioxide (ClO_2) for 15 min resulted in differential expression of 340 genes. These genes were linked to diverse functions, including carbohydrate and glycerol metabolism, protein quality control, homeostasis, PTS system, quorum sensing, and oxidative stress responses. Additionally, the upregulation of DNA repair and protein quality control genes indicated that *L. monocytogenes* actively repaired cellular damage caused by oxidative stress. Similarly, our findings suggest that sanitizer exposure triggers a broad transcriptional response involving multiple stress adaptation pathways, rather than a single, pathway-specific reaction. DNA binding transcription factors play a central role in this process, as they bind to specific

genomic regions and coordinately regulate the expression of multiple gene sets (Gaudet et al., 2021).

Redding et al. (2024) reported that sterile cantaloupe juice induced a complex regulatory response in *L. monocytogenes* biofilms, particularly within membrane transport and signal transduction pathways, including PTS, ABC transporters, and two component system. Their study also showed enrichment of upregulated DEGs associated with the PTS pathway. In contrast, Kang et al. (2019) observed downregulation of PTS and starch/sucrose metabolism when *L. monocytogenes* grew on cantaloupe slices, suggesting that gene regulation can differ depending on the growth environment. Furthermore, Redding et al. (2024) reported upregulation of ABC transporter and acid stress response genes in *L. monocytogenes* biofilms in cantaloupe juice, proposing that these genes facilitate metabolic regulation and adaptation to nutrient and pH gradients within biofilms. Together these findings align with our results and suggest that sanitizer-induced transcriptional changes in *L. monocytogenes* are shaped not only by oxidative stress but also by the physiological state of biofilms and the surrounding environment.

In our study, chlorine exposure led to enrichment of genes associated with the PTS, suggesting that sanitizer stress induces metabolic adaptation. Differential regulation of PTS transporters has been described in *L. monocytogenes* as response to different stresses, such as bile stress and intracellular growth (Arvaniti et al., 2025). Previous research has demonstrated that PTS in *L. monocytogenes* is tightly controlled by alternative sigma factors (Chaturongakul et al., 2011; Wang et al., 2014). Wang et al. (2014) reported that σ^L directly controls the expression of multiple PTS components and therefore influences carbohydrate metabolism. Similarly, Chaturongakul et al. (2011) identified 51 and 169 genes regulated by σ^L and σ^H, respectively, including several genes encoding PTS components. These findings indicate that PTS activity is integrated into global stress

regulatory networks. Therefore, the enrichment of PTS-related genes observed in our study likely reflects that sanitizer exposure may activate alternative sigma factor-mediated metabolic reprogramming, allowing *L. monocytogenes* to optimize energy metabolism and maintain cellular homeostasis under stress.

Previous studies have also shown that most gene regulatory responses to oxidative sanitizers are treatment-specific, with only limited overlap between agents such as chlorine dioxide, hydrogen peroxide, ozone, and plasma-activated water (Rajalingam & Van Haute, 2025). Our results revealed that many transcriptional responses were unique to individual sanitizers, while only a subset of genes was shared.

Furthermore, PAA specifically enriched the GO term phosphate ion transmembrane transport, indicating a distinct strategy for maintaining ionic and metabolic homeostasis under oxidative stress. Phosphate transport systems, such as PhoU, have previously been implicated in *L. monocytogenes* survival under bile and anaerobic conditions, suggesting that phosphate regulation plays a broader role in stress adaptation. (Chakravarty et al., 2021). Because PhoU is known to regulate phosphate uptake, energy balance, and stress tolerance, the enrichment of phosphate transport genes in our study suggests that PAA exposure may activate phosphate-dependent regulatory pathways that promote survival under sublethal oxidative stress.

PAA exposure also enriched ABC transporter pathways in our KEGG analysis; membrane transport systems may play a role in the adaptive response to this sanitizer. ABC transporters in *L. monocytogenes* are known to facilitate the uptake of amino acids and peptides as well as the efflux of toxic compounds (Joseph et al., 2006). Several ABC transporters, including BilE, have been linked to both virulence and stress resistance, particularly in response to oxidative and sanitizer stress (Schauer et al., 2010; Ruiz et al., 2016). Previous studies have shown that oxidizing agents

such as H_2O_2, chlorine dioxide, and plasma-activated water differentially regulate ABC transporters in *L. monocytogenes*, indicating that these systems contribute to adaptation under hostile conditions (Rajalingam & Van Haute, 2025). Previous studies have shown also that oxidative sanitizers can differentially modulate ABC transporters in *L. monocytogenes* depending on the specific compound.

The limited DEG response observed for lactic acid and SDC under a 15s contact time likely reflects differences in mode-of-action kinetics, biofilm penetration, and stress-signaling thresholds compared with oxidative sanitizers. Weak-acid stress, such as that induced by lactic acid, typically depends on time-dependent intracellular acidification - diffusion of the undissociated acid followed by proton release - which may not reach a transcription-activating threshold within 15 s, particularly in biofilms where the extracellular polymeric substance (EPS) matrix and local buffering can attenuate pH fluctuations. Likewise, SDC generally requires longer exposure times for Ag^+ to diffuse through the EPS matrix, bind thiol-rich proteins, and disrupt DNA or protein function; rapid neutralization further shortens its effective contact period. Collectively, these factors suggest that the 15 s sublethal treatments with lactic acid and SDC primarily produced physicochemical or post-translational effects insufficient to trigger broad transcriptional reprogramming within the time frame examined.

While insufficient to activate large-scale transcriptional responses, these sublethal exposures nonetheless exerted measurable effects on cell viability. Although lactic acid and SDC elicited minimal transcriptomic changes, both agents caused an approximately 10% reduction in viable biofilm cells, likely through rapid physicochemical interactions rather than transcriptionally mediated stress responses. Lactic acid can transiently acidify the biofilm surface, leading to membrane disruption and partial protein denaturation, while SDC can rapidly inactivate surface-

associated cells through Ag^+ binding to thiol-containing membrane proteins. However, limited diffusion through the EPS matrix and immediate neutralization restricted deeper penetration, confining damage to the outer biofilm layers. These localized physicochemical effects explain the slight but measurable reduction in cell viability observed after 15 s exposure, without triggering a broader transcriptional response. These findings underscore that even short-term, sublethal sanitizer exposures can inflict localized cellular damage without eliciting global transcriptional responses, potentially allowing surviving biofilm populations to persist and recover under repeated or fluctuating sanitation conditions.

It is also important to consider that the transcriptomic responses observed in this study were characterized under controlled laboratory conditions at 25 °C. *L. monocytogenes* is a psychrotrophic organism that frequently encounters refrigerated processing environments. Growth temperature plays a pivotal role in shaping global gene expression, and exposure to lower temperatures can trigger distinct transcriptional programs associated with cold adaptation, membrane remodeling, and stress resistance (Chan et al., 2007; Muchaamba et al., 2021). Therefore, biofilms formed at refrigeration temperatures may exhibit altered gene expression patterns and potentially different responses to sanitizer exposure. Additionally, interspecies interactions within mixed-species biofilms may further influence *L. monocytogenes* gene regulation through metabolic cooperation (Wang et al., 2024). Coexistence with organisms could modify regulatory networks associated with stress tolerance, EPS synthesis, and antimicrobial resistance (Liu et al., 2024). Within the biofilm matrix, cells are held in close proximity by the EPS, allowing strong cell-to-cell interactions that facilitate cooperation or competition among community members (Schwering et al., 2013). Such spatial organization and intercellular signaling, particularly through quorum-sensing mechanisms, can enhance synergistic behaviors

and collectively increase the resilience of mixed-species biofilms to antimicrobial treatments (Liu et al., 2024).

Conclusion

This study demonstrates that *Listeria monocytogenes* biofilms exhibit distinct adaptive responses to sublethal sanitizer exposure. Oxidative sanitizers, particularly chlorine and PAA, induced extensive transcriptional reprogramming involving stress defense, regulatory, and metabolic pathways, whereas lactic acid and SDC caused limited gene expression changes but approximately a 10% reduction in viable biofilm cells through rapid physicochemical effects.

These findings emphasize that sanitizer chemistry and contact time jointly shape the nature and timing of bacterial stress responses. Oxidative agents can provoke rapid, transcriptome-level changes, whereas lactic acid and SDC may require longer exposure, higher activity, or different temperatures to elicit comparable transcriptional programs. Future studies using time-course RNA-seq, paired proteomics or metabolomics, and EPS characterization would help distinguish immediate damage and neutralization effects from true regulatory adaptation. Collectively, these insights advance current understanding of how sanitizer chemistry influences *L. monocytogenes* adaptation and persistence, providing a foundation for optimizing sanitation practices to prevent survival in food processing environments.

Figures

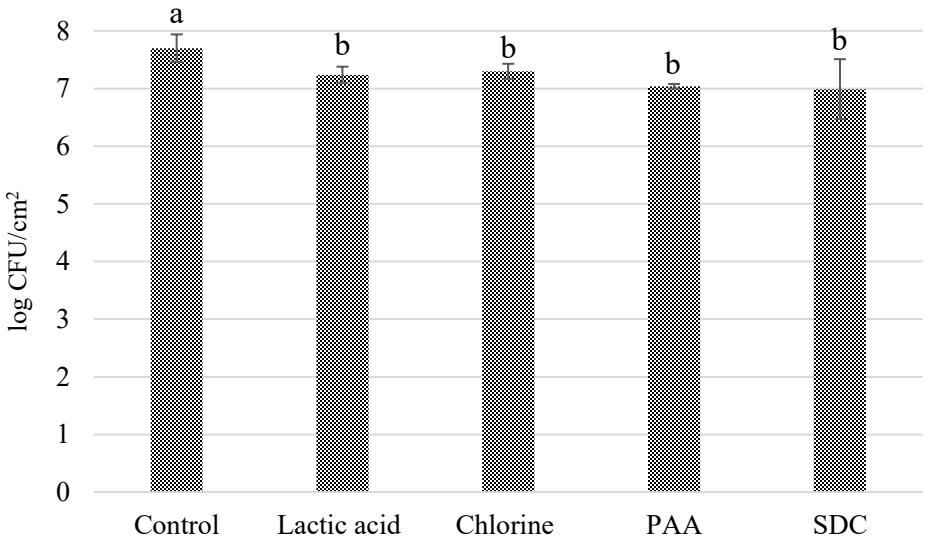


Figure 4.1. Survival of *L. monocytogenes* biofilms after sublethal sanitizer exposure of 0.4% (v/v) lactic acid, 10 ppm chlorine, 10 ppm peracetic acid (PAA), and silver dihydrogen citrate containing 4.85% citric acid and 0.003% silver ions (SDC). Small letters indicate significant differences ($P < 0.05$) among treatments based on one-way ANOVA followed by Tukey's HSD test. Error bars represent standard deviations of three independent replicates.

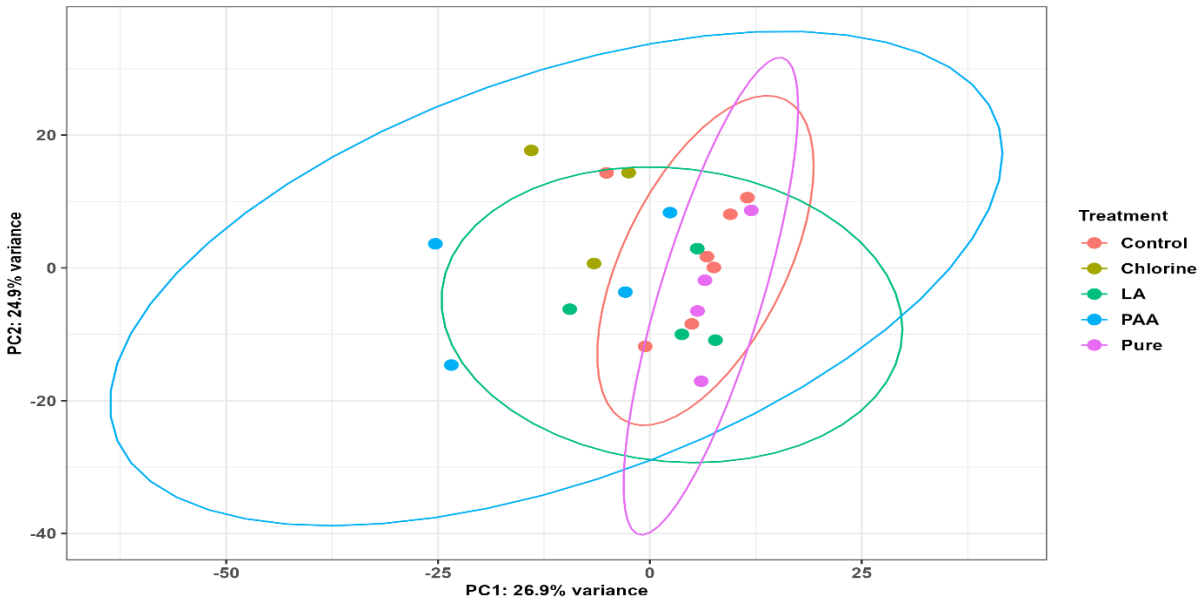


Figure 4.2. Principal component analysis (PCA) plot of *L. monocytogenes* biofilms following exposure to sublethal concentrations of sanitizers and the untreated control. Treatments include 10 ppm chlorine (Chlorine), 0.4% (v/v) lactic acid (LA), 10 ppm peracetic acid (PAA), and silver dihydrogen citrate containing 4.85% citric acid and 0.003% silver ions (Pure). Each point represents an individual sample. Ellipses indicate 95% confidence interval.

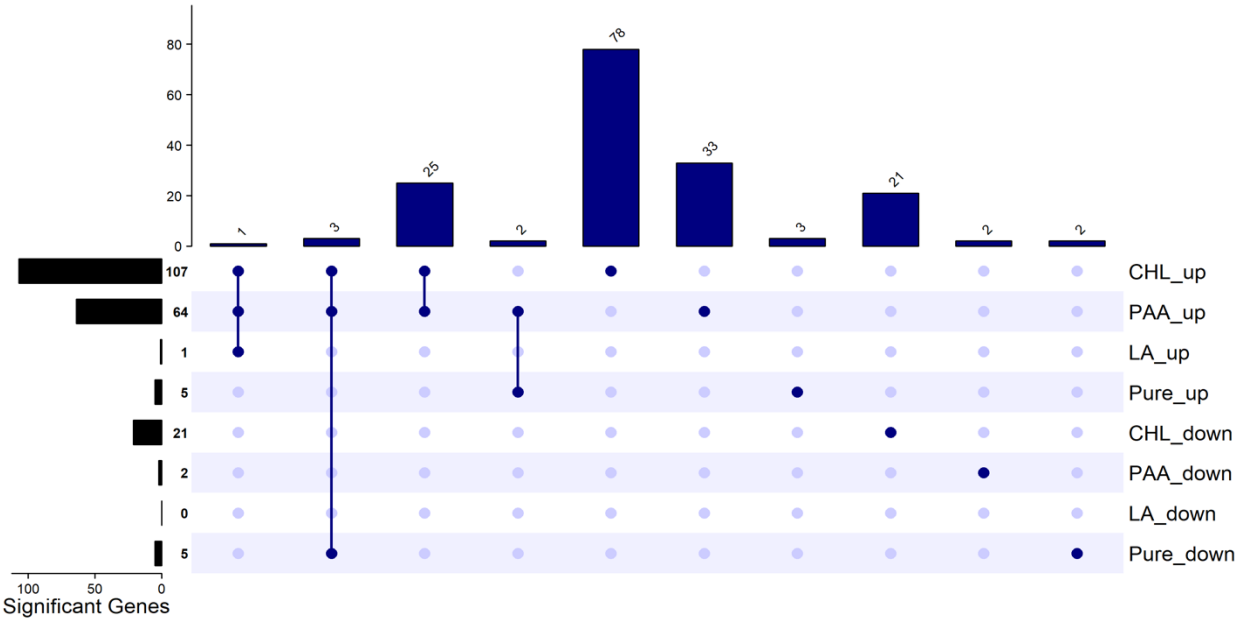


Figure 4.3. UpSet plot illustrating the number of unique and shared differentially expressed genes (DEGs) in *Listeria monocytogenes* biofilms following sublethal sanitizer exposure. Treatments included chlorine (10 ppm), peracetic acid (PAA, 10 ppm), lactic acid (0.4% v/v), and silver dihydrogen citrate (Pure; 4.85% citric acid and 0.003% silver ions). Bars represent the total number

of DEGs within each intersection, while connected dots indicate shared genes between treatments. Horizontal bars on the left show the total number of upregulated and downregulated genes for each sanitizer.

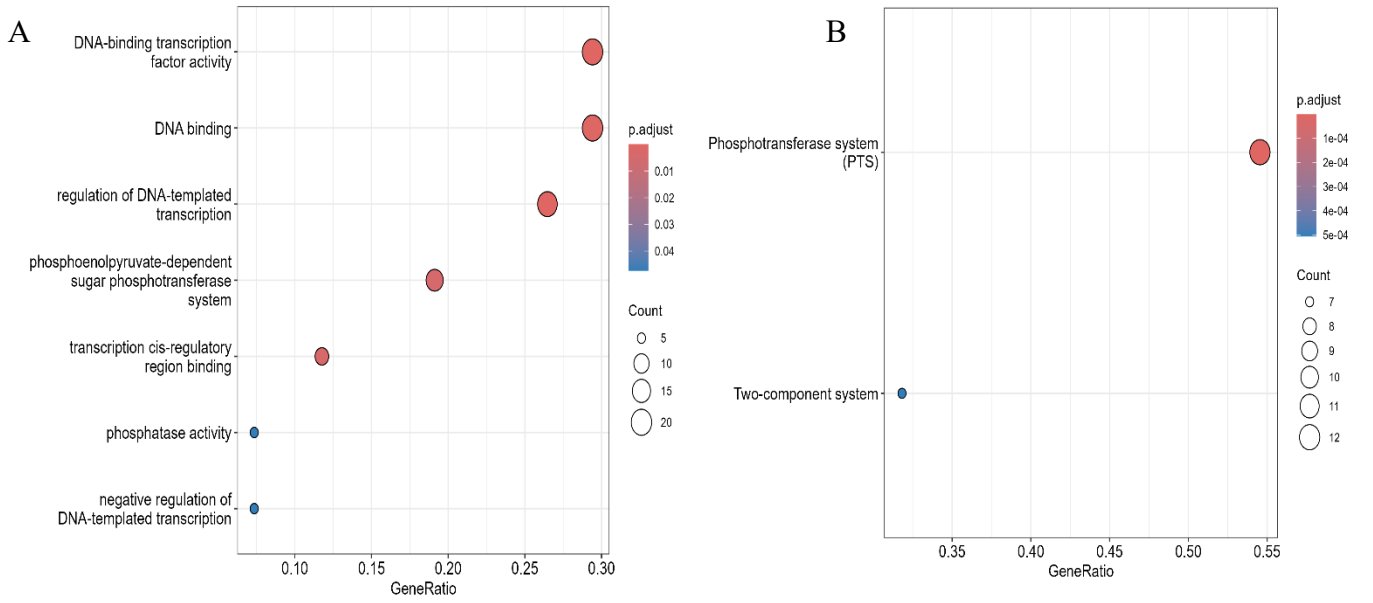


Figure 4.4. (A) Gene Ontology (GO) enrichment of chlorine responsive upregulated genes based on a Benjamini-Hochberg FDR- adjusted p-value < 0.05. Dot size represents the number of genes associated with Go terms. (B) KEGG pathway analysis for genes significantly upregulated in response to 10 ppm chlorine treatment for 15 s.

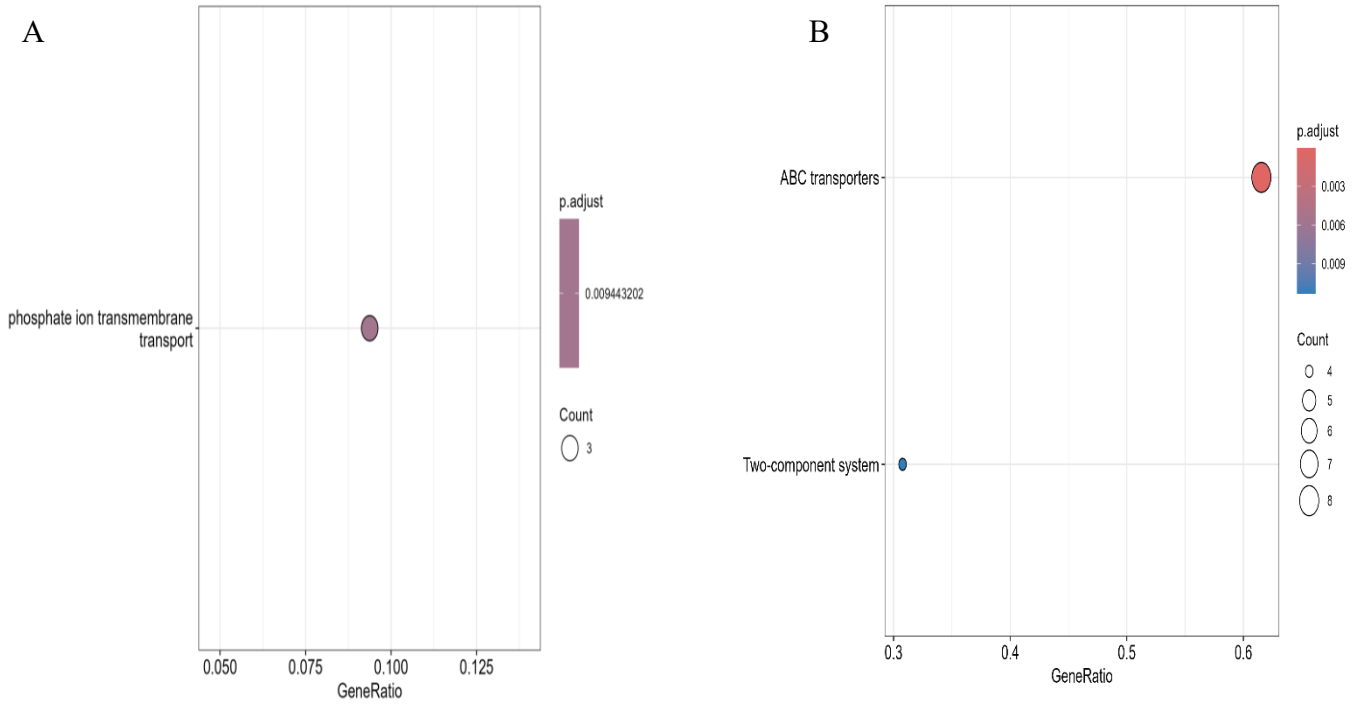


Figure 4.5. (A) Gene Ontology (GO) enrichment of responsive upregulated genes based on a Benjamini-Hochberg FDR- adjusted p-value < 0.05 Dot size represents the number of genes associated with Go terms (B) KEGG pathway analysis for genes significantly upregulated in response to 10 ppm PAA treatment for 15 s.

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Chapter 5 - Evaluation of the effectiveness of commonly used sanitation strategies against dual-species biofilms of *Listeria monocytogenes* and *Pseudomonas fluorescens* in the food industry

Abstract

Multispecies biofilms exhibit enhanced resistance and tolerance to sanitation strategies in the food industry compared to single-species biofilms due to interspecies interactions such as metabolic cooperation, and a robust extracellular polymeric substance (EPS) production. This study evaluated the efficacy of commercially available sanitizers against dual-species biofilms of *Listeria monocytogenes* and *Pseudomonas fluorescens* on various food-contact surfaces. Dual-species biofilms were developed for 96 h at 22 ± 2°C using a Centers for Disease Control and Prevention (CDC) biofilm reactor on stainless steel, polyvinyl chloride (PVC), Teflon, and high-density polyethylene (HDPE) coupons. Treatments included chlorine (500 ppm), peracetic acid (120 ppm), lactic acid (4%) and silver dihydrogen citrate (5%) for 5 min. Scanning electron microscopy (SEM) was conducted to visualize biofilms. Data were analyzed by two-way ANOVA and Tukey's HSD test. Results indicated that all sanitizers significantly reduced *L. monocytogenes* and *P. fluorescens* populations compared to untreated controls ($P < 0.05$). Chlorine was the most effective treatment ($P < 0.05$) resulting in reductions of 3.17, 2.59, 3.33, and 3.72 log CFU/coupon on stainless steel, PVC, HDPE, and Teflon, respectively. Silver dihydrogen citrate demonstrated the least effectiveness ($P > 0.05$), with reductions of 1.49, 1.81, 0.68, and 2.00 log CFU/coupon on the same surfaces. These findings provide insights into how sanitizer type and surface material influence the dual-species biofilm survival, offering guidance for optimizing cleaning and sanitation practices in the food industry.

Introduction

Biofilms pose a significant challenge in the food industry due to their ability to protect microorganisms from environmental stresses and sanitizing agents, leading to persistent contamination of food contact surfaces and serving as a continuous source of food contamination by pathogens and spoilage bacteria (Liu et al., 2023; Voglauer et al., 2025). *Listeria monocytogenes* is particularly concerning because of its capacity to survive and persist in food processing environments for extended periods of time, even under refrigeration and after cleaning or sanitation procedures (Carpentier & Cerf, 2011; Ferreira et al., 2014; Osek et al., 2022). This persistence has been linked to the organism's ability to attach to a wide range of substrates and to coexist within complex multispecies microbial communities (Haddad et al., 2021; Osek et al., 2022).

Previous studies have shown that *L. monocytogenes* does not always form an extensive extracellular polymeric substance (EPS) network when grown in monoculture (Hassan et al., 2004; Kalmokoff & Austin, 2001). However, it can persist and even thrive within multispecies biofilms, where interspecies interactions play a key role in its survival and stress tolerance (Carpentier & Cerf, 2011; Norwood & Gilmour, 2000). *Pseudomonas* species, such as *P. putida* and *P. fluorescens*, are efficient biofilm formers on various industrial surfaces and frequently coexist with *L. monocytogenes* in food processing environments (Flint et al., 2002). These species can modulate the pathogen's attachment, growth, and biofilm development through competitive or synergistic interactions, highlighting the complexity of biofilm communities in industrial settings (Zottola & Sasahara, 1994; Hassan et al., 2004; Puga et al., 2018a). Mixed-species biofilms more accurately reflect real-world conditions, where food processing surfaces are often pre-conditioned by existing microbial communities (Puga et al., 2018b). This pre-conditioning alters surface chemistry,

hydrophobicity, and roughness, which in turn affects the adhesion and colonization of subsequent bacterial species (Van Houdt & Michiels, 2010). Studies have observed that the colonization sequence of *Escherichia coli* O157:H7 and O111:H8 influences the final biofilm composition (R. Wang et al., 2015), while *L. monocytogenes* shows enhanced attachment to pre-formed *P. fluorescens* biofilms compared to simultaneous co-culture (Puga et al., 2018a). Despite these insights, limited information exists regarding how the progression of biofilm development and the environmental stresses typical of food processing settings influence *L. monocytogenes* behavior within multispecies biofilms. Addressing this knowledge gap is critical to understand *L. monocytogenes* persistence in real-world food industry environments and identifying novel strategies for biofilm prevention and eradication.

Furthermore, traditional biofilm model systems used in laboratory research typically rely on submerged surfaces that are continuously exposed to nutrient-rich or flowing media. While these systems provide valuable insights into biofilm growth, structure, and microbial interactions, they may not accurately replicate the conditions encountered in food processing facilities, where surfaces are often intermittently wetted, exposed to condensate, or subjected to nutrient limitation (Somers & Wong, 2004). To better simulate these realistic conditions, our study used the CDC Biofilm Reactor, a standardized and widely validated system developed by the Center for Disease Control and Prevention (Goeres et al., 2005). The CDC Biofilm Reactor allows for controlled shear stress, flow rate, and nutrient conditions to be adjusted, enabling the formation of reproducible biofilms under dynamic environmental parameters that more closely mimic industrial conditions.

Therefore, the main objectives of this research were to evaluate the development of *P. fluorescens* and *L. monocytogenes* biofilms over time on different food contact surfaces, including stainless steel, high-density polyethylene (HDPE), polyvinyl chloride (PVC), and Teflon, and to

assess the efficacy of commercially available sanitizers: 120 ppm peracetic acid (PAA), 500 ppm chlorine, 4% (v/v) lactic acid, and silver dihydrogen citrate (SDC) against dual-species biofilms formed on these surfaces.

Materials and Methods

Bacterial strains and inoculum preparation. *Pseudomonas fluorescens* ATCC 948 (isolated from dairy industry waste) and *Listeria monocytogenes* 390-6 (serotype 1/2a, isolated from a 2011 caramel apple outbreak) were used in this study to form dual-species biofilms. Strains were stored at -80°C in CryoCare Bacteria Preserver beads (Key Scientific, Stamford, TX, USA) until experiments. For inoculum preparation, frozen beads were streaked onto Tryptic Soy Agar (TSA; BD Difco, Sparks, MD, USA) and incubated at 26 ± 2°C for 48 h for *P. fluorescens* and 37 ± 2°C for 24 h for *L. monocytogenes*. A single colony from each plate was picked using a sterile loop and inoculated into 10 ml of fresh Tryptic Soy Broth (TSB; BD Difco, Sparks, MD, USA) and incubated at the optimal growth temperature as specified above, with shaking at 70 rpm. A bacterial 1:1 cocktail was prepared by combining equal volumes of the two overnight cultures. The initial cocktail population of the cocktail was verified by serial dilution in 0.1% peptone water (Bacto, Sparks, MD, USA) and spread plating on TSA for total population, Modified Oxford Agar (MOX; BD Difco, Sparks, MD, USA) for *L. monocytogenes* and Cetrimide Agar (HiMedia Laboratories, Mumbai, India) for *P. fluorescens*.

Coupons materials. Four different food-contact surface materials were selected for this study: stainless steel grade 304, high-density polyethylene (HDPE), polyvinyl chloride (PVC), and Teflon®. Disc coupons (1.27 cm in diameter) of stainless steel 304 and HDPE were purchased from BioSurface Technologies Corporation (Bozeman, MT, USA), while Teflon and PVC were obtained from Sparks Belting (Grand Rapids, MI, USA) and cut into 1.27 cm in diameter by a

Bodor P3015T Fiber Laser cutting machined located at the Kansas State University Technology Development Institute (TDI). Prior to use, all coupons were sterilized by autoclaving followed by a cleaning step: scrubbing with soapy water, sonication at 40 kHz (Branson 3800) in soapy water for 5 min, sonication in tap water for 5 min, and finally sonication in deionized water for 5 min. All coupons were then dried under UV-C light for 15 min.

Biofilm formation assay by crystal violet staining. The biofilm forming ability of each isolate was tested individually following the protocol by Di Ciccio et al. (2022) with minor modifications. Briefly, overnight cultures grown in TSB, were centrifuged at 4,000 rpm for 10 min. Resulting pellets were resuspended in fresh TSB and subsequently diluted in fresh TSB to obtain a final inoculum concentration of approximately 10^6 CFU/ml. Bacterial suspensions (100 µl) were then dispensed into a 96-well polystyrene microplate, with TSB alone used as a negative control. Plates were incubated statically for 24 h at 37 ± 2°C. Following incubation, the contents of the well plates were discarded and washed three times with Phosphate Buffer Saline (PBS; VWR, Solon, OH, USA). After the final wash, biofilms were fixed by incubating the plates at 60º C for 1 h. Bacterial biomass was stained by adding 75 µl of 0.2% w/v crystal violet solution to each well, followed by incubation at room temperature (22 ± 2°C) for 15 min. Well plates were then inverted and gently tapped to remove excess stain solution. Wells were washed three additional times with sterile deionized water. Plates were allowed to dry completely at room temperature. Finally, 75 µl of 95% ethanol was added to each well to solubilize the bound stain, and the absorbance of the solution was measured at 600 nm using a microtiter plate reader (BioTek, Epoch 2, VT, USA). Results were interpreted according to the classification system proposed by Stepanovic et al. (2000) based on our previous study (Deniz et al., 2025).

Dual-species biofilm formation. Dual-species biofilms were grown using a Centers for Disease Control and Prevention (CDC) Biofilm Reactor (BioSurface Technologies, Bozeman, MT, USA) for 96 h at 22 ± 2°C. An aliquot (1 ml) of the bacterial cocktail obtained as described above was used to inoculate a sterile biofilm reactor containing 350 ml of Tryptic Soy Broth (TSB; 20 g/l) prepared in deionized water. The reactor was operated under batch conditions for 24 h with baffle stirring at 60 rpm, as described by Manville et al. (2023). After the batch phase, the reactor was switched to continuous flow for 72 h, during which fresh diluted TSB (1g/l) was supplied at a flow rate of 5 ml/min. To monitor biofilm development over time, coupons were removed from the reactor at 2 h, 24 h and 96 h and transferred into 15 ml centrifuge tubes containing 10 ml of PBS. Biofilm cells were detached by sonication and vortexing for 30 s. This step was repeated three times (ASTM, 2019). Serial dilutions were prepared and enumerated in duplicate on both selective and non-selective media: *P. fluorescens* biofilm cells were enumerated on Cetrimide Agar, *L. monocytogenes* on Modified Oxford Agar (MOX), and total biofilm populations on non-selective Tryptic Soy Agar (TSA).

Antimicrobials applications. In another set of experiments, the efficacy of commercially available sanitizers against mature dual-species biofilms (96 h incubation) formed on coupons was evaluated. The sanitizers tested included 500 ppm free liquid chlorine (Diversey, Fort Mill, SC), with the concentration confirmed using a chlorine test kit (ChemWorld, Kennesaw, GA); 120 ppm peracetic acid (PAA) (SaniDate 15.0, BioSafe Systems, LLC; East Hartford, CT), confirmed using a peracetic acid test kit (ChemWorld, Kennesaw, GA); 5% Silver dihydrogen citrate (SDC; Pure Bioscience, El Cajon, CA, USA), used as a ready-to-use solution, and 4% lactic acid (Purac® Corbion, Blair, NE), with the concentration measured using a lactic acid test kit (ChemWorld, Kennesaw, GA).

Liquid sanitizers were applied using an immersion method. Each bioreactor rod containing 3 coupons was aseptically removed from the reactor and immersed into a centrifuge tube containing 30 ml of the target sanitizer solution for 5 min without agitation.

Cell recovery and microbial enumeration. After treatments, coupons were immediately removed from the rods and transferred into 15 ml centrifuge tubes containing10 ml of the appropriate neutralizer. For PAA and chlorine treatments, double-strength D/E broth was used, whereas lactic acid and SDC treatments were neutralized by single-strength D/E broth. To ensure complete neutralization, a neutralizing assay was performed for each sanitizer (Chen, 2011). Fully attached cells were recovered as explained above and serial dilutions performed on TSA for total counts, MOX Agar for *L. monocytogenes*, and Cetrimide Agar for *P. fluorescens* enumeration. Colonies were counted and reported as log CFU/coupon.

Microscopy analysis. Scanning electron microscopy (SEM) was used to visualize clean coupons (without biofilms), mature (96 h) single-species biofilms of *P. fluorescens*, *L. monocytogenes,* and dual-species biofilms. Biofilm fixation was performed on colonized coupons using 4% methanol-free formaldehyde solution (Cell Signaling Technology, USA) for 15 min at room temperature, following the protocol of Chaggar et al. (2024). Coupons were then mounted on aluminum stubs with carbon tape and sputter-coated with a thin gold layer (4 nm) using the SPI-Module Sputter Coater (SPI Supplies, West Chester, PA). Samples were examined with a Hitachi S4700 II Cold Field Emission Scanning Electron Microscope (Hitachi High Technologies), equipped with both lower and upper secondary electron detectors. Imaging was performed in Ultra-High-Resolution mode under the following parameters: accelerating voltage of 3–5 kV, condenser lens setting, emission current of 5–10 μA, 50 μm aperture (aperture 2), working distance of 12.0 ± 1 mm, and acquisition resolution of 2560 × 1920 pixels with ~40 s scan speed.

Both sputter coating and SEM imaging were conducted at the University of Kansas Microscopy and Analytical Imaging Research Resource Core Laboratory (Lawrence, KS, USA).

Experimental design and statistical analysis. Sanitizer treatments were randomized across rods in the CDC biofilm reactor, and experiments were conducted in triplicate for each treatment condition. The treatment structure followed a factorial arrangement with three microbial population types (*L. monocytogenes* alone, *P. fluorescens* alone, and *L. monocytogenes* and *P. fluorescens* combined), five sanitizer treatments (PAA, chlorine, SDC, lactic acid, and an untreated control), four surface materials (stainless steel, PVC, HDPE, and Teflon), and three observation times (2 h, 24 h, 96 h after inoculation). The study used an incomplete block design, with the CDC biofilm reactor serving as the blocking factor. Each bioreactor contained 16 coupons, which consistently included all four surface materials under four different sanitizer-time combinations. Because the CDC bioreactors could not accommodate all treatments simultaneously, each bioreactor included one control treatment (i.e., no sanitizer) evaluated at 96 h to allow comparisons across runs. In addition, each run also included a control measured at either 2 h or 24 h. The remaining coupons were randomly assigned to two of the four sanitizer treatments. Microbial counts of *P. fluorescens*, *L. monocytogenes*, and total populations were analyzed separately using linear mixed models, with material, treatment, and their interaction specified as fixed effects and the CDC biofilm reactor treated as a random effect. Analysis of variance (ANOVA) models were used to further analyze the impact of the treatment effects on the observed variations in the population growth of bacteria under different scenarios. Multiple comparisons with Tukey adjustments were evaluated with a significance level of $\alpha = 0.05$. All statistical analyses were performed using R (version 4.4.0, R Foundation for Statistical Computing, Vienna, Austria).

Results

Biofilm formation ability. Figure 5.1 shows the different biofilm production levels of single and dual-species biofilms formed by the strains used in this study. This assay was conducted to understand interspecies interactions during biofilm formation, since strong biofilm formation might contribute to increased resistance to cleaning and sanitation.

According to this classification, both *P. fluorescens* and *L. monocytogenes* were found to be biofilm formers but with different capacities. When cultured individually, *P. fluorescens* produced the greatest biomass, while *L. monocytogenes* showed significantly lower biomass production ($P < 0.05$). Nevertheless, when multi-species biofilms were formed, biomass production was greater than *L. monocytogenes* alone ($P < 0.05$).

Development of dual-species biofilms. The growth of *L. monocytogenes* and *P. fluorescens* during biofilm formation was evaluated over a 96 h incubation period on different food-contact surfaces (Figure 5.2). Stainless steel and HDPE surfaces followed a similar trend (Figure 5.2A and 2B), with *L. monocytogenes* counts significantly higher than those of *P. fluorescens* across all sampling points ($P < 0.05$). On PVC (Figure 5.2C), the *L. monocytogenes* population was also consistently higher than *P. fluorescens* over time ($P < 0.05$), except at 24 h when the two counts were comparable ($P > 0.05$). Conversely, on Teflon (Figure 5.2D), neither species showed a difference in count over the first 2 sampling points ($P > 0.05$), but the *L. monocytogenes* population was ultimately higher by the end of the maturation period (96 h; $P < 0.05$). Overall, *L. monocytogenes* exhibited significantly higher cell population counts over time compared to *P. fluorescens* across all tested surfaces by the end of experiment (96 h; $P < 0.05$).

Effect of sanitizer treatment on the dual species biofilms. After assessing the growth of dual-species biofilms on the different food-contact surfaces, the effectiveness of commercial

sanitizers against biofilm was evaluated. Table 5.1 displays the main effects and interactions of material and sanitizer on dual-species biofilms. Both material and treatment, as well as their interaction, had significant effects on microbial recovery ($P < 0.05$) on dual-species biofilm as well as single strain. These results indicate that sanitizer efficacy varied depending on the surface material on which biofilms were formed.

All sanitizers tested in this study resulted in significantly lower recovered cell counts ($P < 0.05$) of *L. monocytogenes*, *P. fluorescens,* as well as their total combined counts compared to untreated controls (Table 5.2). Among sanitizers, chlorine was the most effective showing enhanced effectiveness on HDPE, with a remaining population of 5.30 ± 0.19 log CFU/coupon) and stainless steel (5.26 ± 0.09 log CFU/coupon). Remaining populations after chlorine treatment on PVC (7.06 ± 0.09 log CFU/coupon) and Teflon (5.58 ± 0.17 log CFU/coupon; $P < 0.05$) were higher ($P < 0.05$). Similar observations were recorded when *L. monocytogenes* and *P. fluorescens* were enumerated individually ($P < 0.05$) after chlorine treatment.

PAA also showed significant ($P < 0.05$) effectiveness against dual-species biofilm: total biofilm counts were significantly reduced on all tested surfaces compared to untreated controls ($P < 0.05$). On HDPE, PAA treatment resulted in the greatest ($P < 0.05$) reduction for total counts, with a remaining population of 6.27 ± 0.15 log CFU/coupon, while single species counts were 5.41 ± 0.57 and 5.02 ± 0.57 log CFU/coupon for *L. monocytogenes* and *P. fluorescens*, respectively.

SDC treatment also led to a significant reduction in total biofilm populations compared to their respective untreated controls ($P < 0.05$) on all tested surfaces except Teflon. On Teflon, recovered populations of *L. monocytogenes* remained at 7.95 ± 1.65 log CFU/coupon and did not differ from the untreated control ($P > 0.05$). Nevertheless, *P. fluorescens* was significantly reduced after

treatment ($P < 0.05$). Overall, SDC was the least effective sanitizer compared to the other treatments to reduce dual-species biofilm counts on the surface materials evaluated in this study.

Lactic acid also demonstrated significant antimicrobial activity ($P < 0.05$) against dual-species biofilms, with recovered total counts significantly lower than untreated controls on all tested surfaces ($P < 0.05$). On HDPE, lactic acid was particularly effective, reducing both *L. monocytogenes* and *P. fluorescens* populations by approximately 3.5 log CFU/coupon compared to untreated controls ($P < 0.05$). In contrast, higher counts were observed on PVC and Teflon, indicating lower efficacy of this sanitizer when biofilms were grown on these surfaces.

Microscopy analysis. Figure 5.4 illustrates the clean, biofilm free surface (Figure 5.4A) followed by colonization of HDPE when single-species of *L. monocytogenes* biofilms are formed (Figure 5.4B), *P. fluorescens* single-species biofilms (Figure 5.4C), and dual-species biofilms formed by both organisms (Figure 5.4D). *L. monocytogenes* preferentially colonized the crevices and folds of the surface (Figure 5.4B), while single species *P. fluorescens* biofilms showed greater surface coverage and structural integrity (Figure 5.4C). Dual-species biofilms developed instead into denser, multilayered communities (Figure 5.4D) and more extensive surface coverage, suggesting that the interaction between these bacteria improve the structural robustness of the biofilm. Dual-species biofilms appeared as elongated rod-shaped structures embedded within the extracellular matrix, forming compact networks. Nevertheless, enumeration consistently indicated higher *L. monocytogenes* counts in dual-species biofilms, suggesting the organism's ability to thrive in complex microbial communities.

Discussion

Pseudomonas fluorescens is commonly found in food processing environments, where it increases the risk of product spoilage and cross-contamination (Alexandre et al., 2025; Puga et al.,

2018a). Biofilm formation by *P. fluorescens* is of particular concern in the food industry, as it can potentially provide a protective reservoir for foodborne pathogens such as *Listeria monocytogenes* (Diaz et al., 2025; Thomassen et al., 2023; Tirpanci Sivri et al., 2023).

In our study, we observed an enhanced biofilm production when *L. monocytogenes* and *P. fluorescens* were grown together to form dual-species biofilms. Previous studies have suggested that *L. monocytogenes* attachment to surfaces in food-processing environments may be promoted by *P. fluorescens* pre-establishment and formation of biofilms. *P. fluorescens* produces a large amount of EPS that facilitates adhesion and colonization by *L. monocytogenes* (Puga et al., 2018a). In the present study, *P. fluorescens* showed greater biofilm production than *L. monocytogenes*. When biofilms were formed in dual-species, the biofilm formation of *L. monocytogenes* was enhanced compared to single-species biofilms, as determined by crystal violet staining, possibly due to increased production of extracellular polysaccharides. A similar observation was reported by Lauer Cruz & De Souza Da Motta (2019), where the *P. fluorescence* PL5.4 strain was identified as a larger EPS producer compared to *L. monocytogenes* ATCC 7644. Similarly, Maifreni et al. (2023) classified the *P. fluorescens* strain Ps_19 as a strong biofilm producer regardless of the temperature at which the biofilms was developed.

Haddad et al. (2021) also evaluated dual-species biofilm formation using a CDC biofilm reactor on stainless steel and HDPE coupons under simulated meat processing conditions. By day 5, *L. monocytogenes* biofilm cell numbers reached 8.1 log CFU/cm^2 on HDPE and 7.6 log CFU/cm^2 on stainless steel, while *P. fluorescens* counts were comparable across the two surfaces (7.0 and 7.2 log CFU/cm^2 on HDPE and stainless steel, respectively). In our study, *L. monocytogenes* populations were slightly higher on both HDPE (8.60 ± 0.28 log CFU/coupon) and stainless steel (8.61 ± 0.33 log CFU/coupon) compared to the values reported by Haddad et al.

(2021), whereas *P. fluorescens* populations were lower, at 6.74 ± 0.49 and 6.22 ± 0.63 log CFU/coupon, respectively. While overall values were comparable between the two studies, lower *P. fluorescens* counts in our assay could reflect differences in biofilm formation media and nutrient availability. Haddad et al. (2021) used a meat-slurry-based medium that simulated nutrient residues in meat processing environments, whereas we used a general purpose growth medium. Zhou et al. (2024) instead assessed *L. monocytogenes* and *P. fluorescens* biofilm formation under static conditions with a 1:1 inoculation ratio. The authors observed significantly higher populations of *P. fluorescens* ($P < 0.05$) after 72 h at 25 °C. Similarly, Ripolles-Avila et al. (2022) reported that when *L. monocytogenes* and *P. fluorescens* were grown together on stainless steel coupons under static conditions, counts reached 5.79 log CFU/cm^2 after 72 h. These findings highlight how growth conditions, including temperature, nutrient composition, and surface properties strongly shape the competitive and synergistic dynamics between *L. monocytogenes* and *P. fluorescens*. These environmental factors may also modulate cooperative interactions, as the two species can exchange metabolites or signaling molecules that enhance biofilm formation and persistence. Such interactions could involve quorum-sensing signals or metabolic byproducts that promote mutual growth within mixed-species biofilms. The differences reported could also be attributed to strain-specific characteristics, as some *L. monocytogenes* strains are known to be stronger colonizers than others, as well as differences in biofilm-formation protocols (dynamic vs static). Pant et al. (2025) observed that the presence of *P. fluorescens* enhanced *Listeria* population in a CDC biofilm reactor, as reported in our study.

In our experiments, sanitizer treatments reduced but did not eliminate *P. fluorescens – L. monocytogenes* dual-species biofilms, as indicated by surviving populations. Factors contributing to multispecies biofilm tolerance include reduced diffusion of antimicrobials into the biofilm

matrix and physiological alterations of the cells (Rolon et al., 2024). As reported by Stewart (2015), antimicrobial penetration depends on biofilm thickness, effective diffusivity, agent reactivity, sorptive capacity of the matrix, the applied concentration and the exposure time of the antimicrobial agents.

Four different sanitizers commonly used in the food industry were tested in our research. Chlorine-based sanitizers are widely used in the food industry because of their availability, low costs, and strong antimicrobial activity (Maifreni et al., 2023; Rodríguez-López et al., 2022). Chlorine is a potent oxidant that damages cell membranes and liberates proteins, RNA, DNA, and enzymes, ultimately leading to bacterial inactivation (Thi Nguyen et al., 2024). Contrasting results have been reported in the literature. Chen & Stewart (1996) observed the limited penetration of chlorine into *Pseudomonas aeruginosa* biofilms. Maifreni et al. (2023) instead showed that *P. fluorescens* biofilms formed on stainless steel and polytetrafluoroethylene (PTFE) were largely sensitive to chlorine and PAA-based sanitizers. Wang et al. (2018) reported that treatment with 200 mg/l sodium hypochlorite reduced 5-day-old *P. fluorescens* biofilms on stainless steel from 8 to 5 log CFU/cm^2. In the present study, we observed that treatments with free liquid chlorine (500 ppm for 5 min) were the most effective among the sanitizer strategies tested. Norwood & Gilmour (2000), however, reported limited efficacy in 28-day old multispecies biofilms of *L. monocytogenes*, *P. fragi*, and *Staphylococcus xylosu*, with only a 2 log CFU/plug reduction for *L. monocytogenes* after 20 min exposure to 1000 ppm free-chlorine. Pant et al. (2025) also observed modest chlorine performance, reporting a reduction of 0.1 log CFU/cm^2 for *P. fluorescens* and 1 log CFU/cm^2 reduction of *L. monocytogenes* biofilms grown in a CDC reactor when treated with sodium hypochlorite at 50 ppm for 5 min. The greater reductions observed in our study may be explained by the higher chlorine concentration and the resulting oxidative activity against biofilm

cells, as well as differences in chlorine source, since free chlorine levels directly influence antimicrobial efficacy (CDC, 2023). In addition, the presence of other microbial species can alter antimicrobial susceptibility.

Other sanitizers such as PAA, quaternary ammonium compounds (QACs), iodine compounds, and lactic acid are also used in food processing environments and have demonstrated good antimicrobial activity against bacteria (Tirpanci Sivri et al., 2023). Fatemi & Frank (1999) evaluated 48 h old biofilms of *L. monocytogenes* and *Pseudomonas* spp. treated with PAA, peroctanoic acid (POA), and chlorine, with or without organic load and reported that peracid sanitizers performed better than chlorine, and POA was more effective than PAA in inactivating *Pseudomonas* but not *Listeria* after 1 and 5 min of treatment. In our study, PAA treatment also resulted in significant reductions ($P < 0.05$) of dual-species biofilms across all tested surfaces, although reductions in *P. fluorescens* were generally lower than those observed for *L. monocytogenes.* Previous studies have shown that many *Pseudomonas* spp. harbor multidrug-efflux-pump systems, which can reduce sanitizer susceptibility and contribute to biofilm persistence (Thomassen et al., 2023).

The efficacy of SDC was also evaluated against dual-species biofilms. SDC is a stabilized silver ion complex with antimicrobial activity and previous studies have reported its efficacy against biofilms and viruses (Buckley et al., 2018; Ivers et al., 2024; Manuel et al., 2017; Masuku et al., 2014). Masuku et al. (2014) has reported reductions up to 5 log CFU/cm^2 in adhered *Listeria* on aluminum and stainless coupons following 2 min of SDC exposure. Manville et al. (2023) observed up to 3 log CFU/cm^2 reductions against *L. monocytogenes* biofilms. In our study, SDC treatment of dual-species biofilms resulted in significant reductions as compared to untreated

controls, although its overall activity was limited relative to other sanitizers. To our knowledge, this is the first study to assess its efficacy against dual-species biofilms.

Lactic acid is another sanitizer used in the food industry for surface decontamination and has shown strong antimicrobial activity (Byun et al., 2022; Nkosi et al., 2021). This sanitizer reduces the intracellular pH and disrupts the proton concentration gradient across the inner membrane, thereby impairing essential cellular functions (Byun et al., 2022; Wang et al., 2015). Its efficacy against single-species biofilms on food-contact surfaces has been studied (Deniz et al., 2025; Manville et al., 2023; Kostoglou et al., 2024; Lindsay et al., 2022). Kostoglou et al. (2024) demonstrated that lactic acid inhibited the formation of mixed-culture biofilms of *Campylobacter* spp. on stainless steel and polystyrene by more than 2 log CFU/cm^2 at 0.34% v/v. In contrast, the authors reported that the pre-formed biofilms (~ 6 -7 log CFU/cm^2) could not be eradicated even when lactic acid was applied at concentrations exceeding 5% v/v, indicating that established biofilms remained highly tolerant to treatment. Similarly, in our study, 4% lactic acid demonstrated only limited efficacy against dual-species biofilms. Other studies have also shown that the performance of lactic acid-based sanitizers is strongly dependent on concentration and target organism (Arias-Moliz et al., 2012; Lindsay et al., 2022).

Scanning electron micrographs revealed greater complexity and denser structure in dual-species biofilms as compared to surfaces colonized by single species. Haddad et al. (2021) reported extensive surface coverage and multilayered cells with EPS production on HDPE surfaces by *L. monocytogenes* and *P. fluorescens*, supporting our findings. In comparison to the SEM images presented by Dong et al. (2022) for *L. monocytogenes–P. aeruginosa* biofilms in chicken juice at 24 h, our results showed denser structures with more extensive matrix formation, highlighting the role of species interactions and growth conditions in shaping biofilm architecture.

Conclusion

This study provides novel insights into the efficacy of commercial sanitizers against dual-species biofilms formed under dynamic conditions. *P. fluorescens* was found to be a strong biofilm former. Nevertheless, *L. monocytogenes* was able to grow in the presence of *P. fluorescens*, reaching higher population counts under dynamic conditions. All sanitizers tested demonstrated significant efficacy against dual-species biofilms but did not achieve complete eradication. Future studies should focus on elucidating the mechanisms of tolerance in dual-species biofilms and evaluating conditions that enhance sanitizer efficacy.

Tables and Figures

Table 5.1. Statistical analysis (ANOVA) showing the individual and interaction effects of surface material and sanitizer treatments on dual-species biofilms of *L. monocytogenes* and *P. fluorescens.*

Parameters	**P-value**		
	L. monocytogenes	*P. fluorescens*	Total counts (*L. monocytogenes* + *P. fluorescens*)
Material	< 0.05*	< 0.05*	< 0.05*
Treatment	< 0.05*	< 0.05*	< 0.05*
Material x Treatment	< 0.05*	< 0.05*	< 0.05*

Values marked with * letters denote statistical significance with $P < 0.05$.

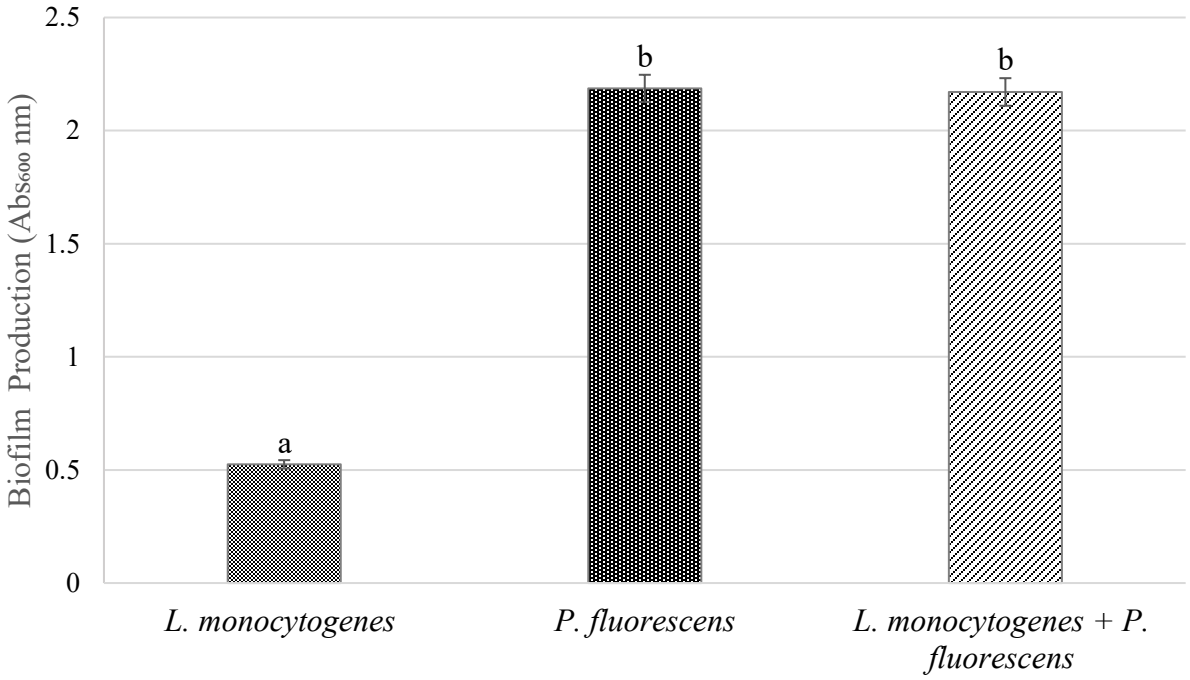
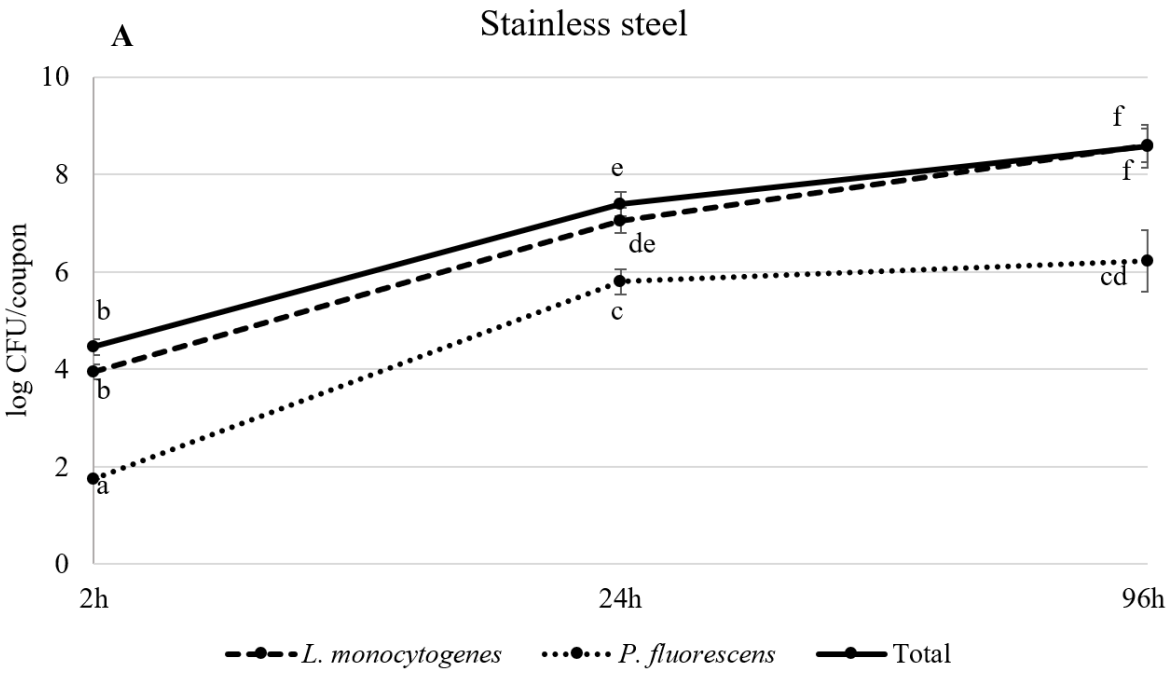
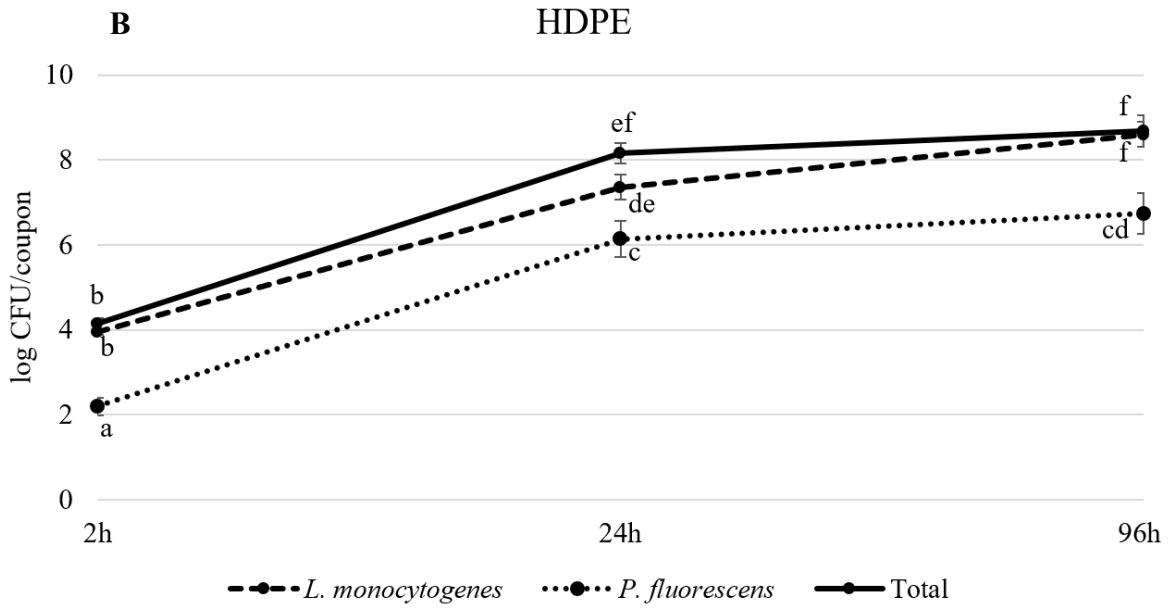


Figure 5.1. Biofilm production by *L. monocytogenes*, *P. fluorescens* as single species and dual species (*L. monocytogenes* + *P. fluorescens*), measured by crystal violet staining. Average absorbance (600 nm) is represented by column and standard error by bars. Small letters indicate statistical differences in biofilm production among strains.


A
Stainless steel
log CFU/coupon
10
8
6
4
2
0
2h
24h
96h
a
b
b
c
de
e
cd
f
f
L. monocytogenes
P. fluorescens
Total


B
HDPE
log CFU/coupon
10
8
6
4
2
0
2h
24h
96h
a
b
b
c
de
ef
cd
f
f
L. monocytogenes
P. fluorescens
Total

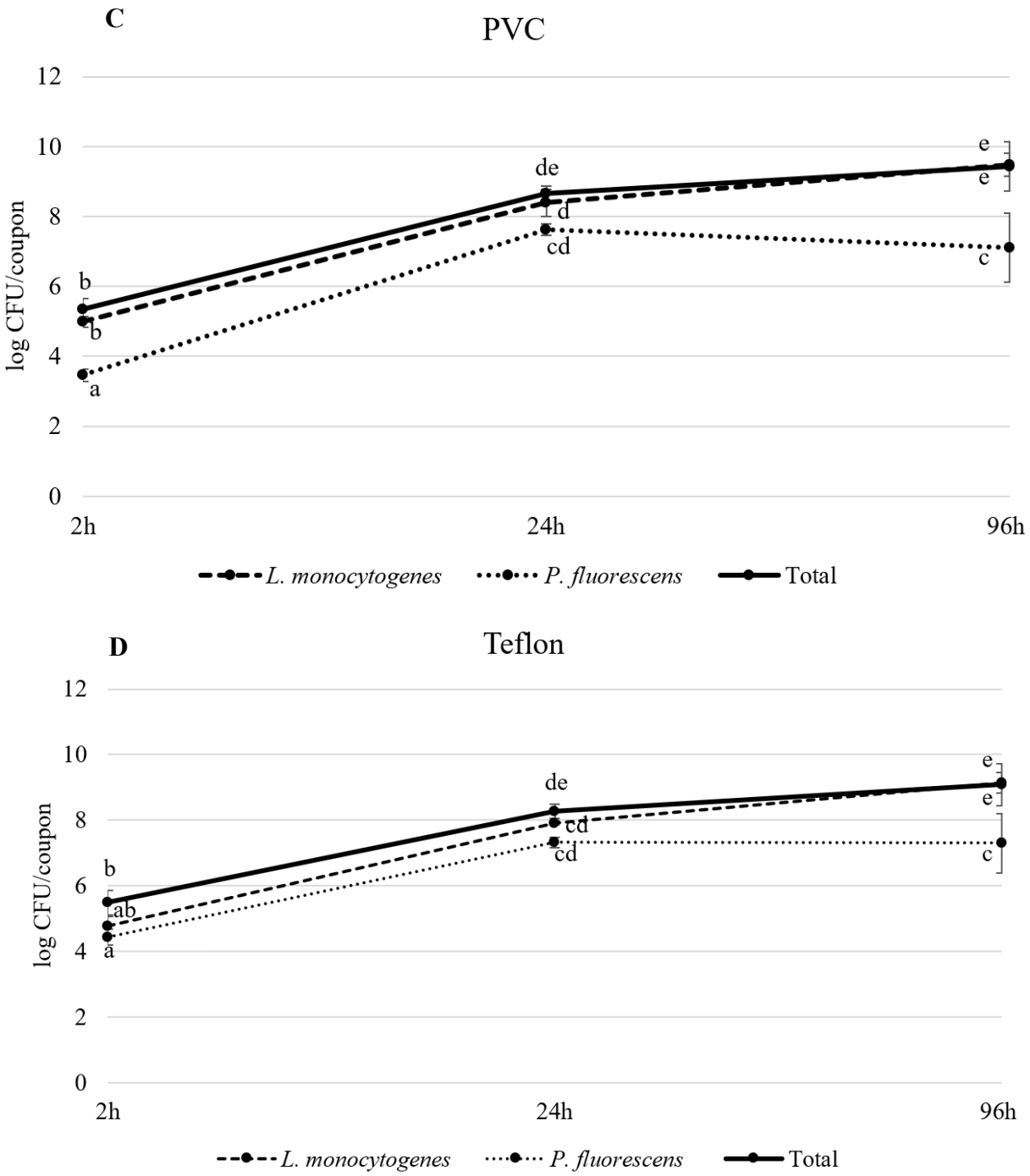


Figure 5.2. Enumeration of *L. monocytogenes*, *P. fluorescens* and total biofilm population (Log CFU/coupon) over time (2 h, 24 h, and 96 h) on A) stainless steel, B) HDPE, C) PVC, and D) Teflon. Lowercase letters indicate significant differences ($P < 0.05$) among microbial counts across time points on the same type of surface.

Table 5.2. Microbial populations (log CFU/coupon ± standard deviation) of *L. monocytogenes* and *P. fluorescens* and dual-species (*L. monocytogenes* + *P. fluorescens*) recovered from different surface materials following treatment with sanitizers.

Bacteria	Treatment	Stainless Steel	PVC	HDPE	Teflon
L. monocytogenes	PAA	6.22 ± 0.36 c	7.87 ± 0.52 c	5.41 ± 0.57 c	7.62 ± 0.40 b
	Chlorine	4.41 ± 0.24 e	6.31 ± 0.09 d	4.4 ± 0.17 d	5.05 ± 0.03 d
	Silver Dihydrogen citrate	6.88 ± 0.69 b	8.64 ± 0.46 b	6.14 ± 0.28 b	8.65 ± 0.59 a
	Lactic acid	5.34 ± 0.54 d	7.3 ± 0.36 c	4.96 ± 0.26 cd	5.83 ± 0.21 c
P. fluorescens	PAA	5.15 ± 0.08 b	5.45 ± 0.13 b	5.02 ± 0.57 b	5.43 ± 0.04 b
	Chlorine	4.54 ± 0.43 bc	5.87 ± 0.09 b	3.95 ± 0.31 bc	5.10 ± 0.09 b
	Silver Dihydrogen citrate	3.61 ± 0.30 c	5.55 ± 0.47 b	3.72 ± 0.24 c	4.33 ± 0.46 b
	Lactic acid	4.09 ± 0.60 bc	5.06 ± 0.07 b	3.23 ± 0.21 c	4.96 ± 0.28 b
L. monocytogenes + *P. fluorescens*	PAA	7.06 ± 0.23 b	8.27 ± 0.10 b	6.27 ± 0.15 b	8.12 ± 0.37 b
	Chlorine	5.26 ± 0.09 c	7.06 ± 0.09 c	5.30 ± 0.19 b	5.58 ± 0.17 d
	Silver Dihydrogen citrate	6.94 ± 0.99 b	7.84 ± 0.92 bc	7.95 ± 1.65 a	7.24 ± 0.91 bc
	Lactic acid	6.19 ± 0.07 bc	7.43 ± 0.24 bc	5.61 ± 0.30 b	6.46 ± 0.28 cd

[*] Untreated mature biofilms (controls) yielded the following average populations (log CFU/coupon ± standard deviation): for *L. monocytogenes*: 8.61 ± 0.33, 9.48 ± 0.32, 8.6 ± 0.28, and 9.14 ± 0.32 on stainless steel, PVC, HDPE, and Teflon, respectively; *P. fluorescens* 6.22 ± 0.63, 7.11 ± 0.99, 6.74 ± 0.49, and 7.30 ± 0.91 on stainless steel, PVC, HDPE, and Teflon, respectively; and total counts of *L. monocytogenes* and *P. fluorescens* on non-selective media 8.43 ± 0.32, 9.65 ± 0.25, 8.63 ± 0.34, and 9.30 ± 0.24 on stainless steel, PVC, HDPE, and Teflon, respectively. Statistical comparisons were conducted within each bacterial group and surface material by treatment.

Different lowercase letters indicate significant differences ($P < 0.05$) among treatments within each bacterial group and surface material.

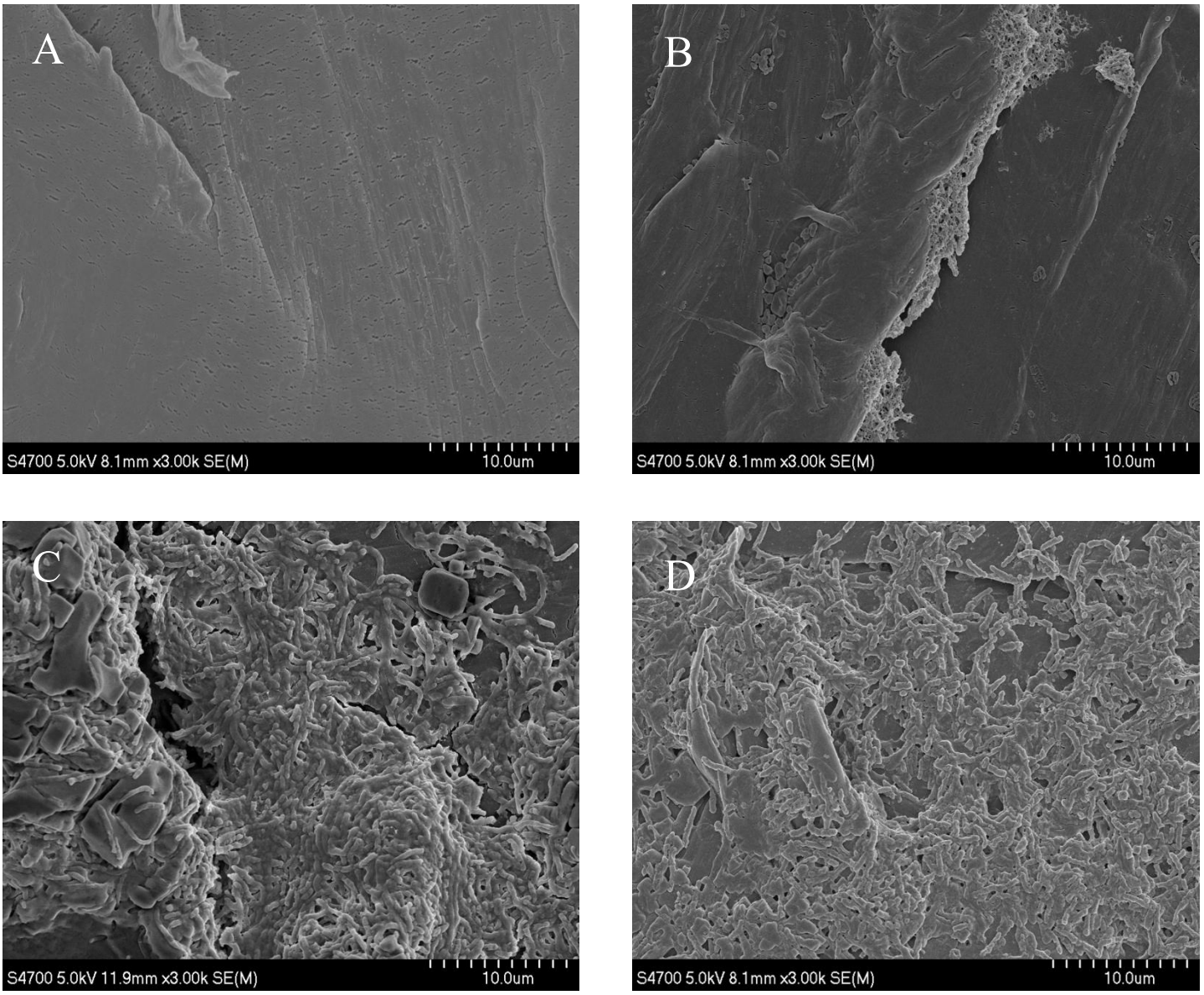


Figure 5.3. Scanning electron microscopy (SEM) images of A) clean HDPE coupon (no microbial growth), B) single-species biofilms *of Listeria monocytogenes* 390-6, C) single-species biofilms of *Pseudomonas fluorescens* ATCC 948 , and D) dual-species biofilms formed by *L. monocytogenes* and *P. fluorescens* on HDPE coupons.

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Chapter 6 - Conclusions

The ability of *Listeria monocytogenes* to adhere to hard surfaces and form biofilms presents a major concern for food safety and public health, as biofilms are more resistant to cleaning and sanitation than planktonic cells. In produce processing environments, biofilms often exist as multiple species communities that include spoilage microorganisms such as *Pseudomonas fluorescens*, further complicating their control and removal.

This research demonstrated that the type of surface material influences biofilm formation, architecture, and sanitizer efficacy. Variations among different surfaces affected both the extent of biofilm development and the efficiency of antimicrobial treatments. These findings highlight the importance of evaluating biofilm behavior and sanitizer performance on surfaces commonly used in the produce industry to improve sanitation strategies.

Transcriptomic analysis further showed that different sanitizers induce distinct stress response pathways in *Listeria monocytogenes*, providing valuable insights into the mechanisms underlying bacterial survival. The differential expression of genes related to metabolic reprogramming suggests that *L. monocytogenes* activates multiple adaptive strategies to survive under sanitizer-induced stress.

Moreover, the dual-species biofilm study revealed that *L. monocytogenes* can coexist and proliferate within mixed biofilms formed with *P. fluorescens*. The presence of *P. fluorescens* enhanced the overall biofilm mass (Chapter 5) as compared to *L. monocytogenes* single-species biofilms (Chapter 3).

While this work provides important mechanistic insights into biofilm formation and sanitizer response, it was conducted under controlled laboratory conditions and does not fully replicate the complex environmental parameters of produce processing facilities. Factors such

as temperature fluctuations, presence of food residues, humidity, and prolonged exposure to sublethal sanitizer concentrations may further influence biofilm development and persistence. Additionally, biofilms in real processing environments can remain on surfaces such as drains, gaskets, or drip pans for extended periods where they are continuously exposed to combinations of nutritional, chemical, and mechanical stresses not assessed in this study.

Future research should therefore incorporate more environmentally realistic conditions, including chilled processing temperatures, organic residue loads, and long-term biofilm aging, to better model the survival and resilience of *L. monocytogenes* in produce environments. Integrating such conditions with omics-based analyses will further improve our understanding of *L. monocytogenes* adaptation and inform more effective, evidence-based sanitation and monitoring programs.