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Title: Efficacy of Sanitizers at Controlling *Listeria* spp. and *Listeria monocytogenes* When Challenged with Variables Commonly Encountered in Food Processing Environments

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Listeria monocytogenes (*Lm*) is a pathogenic bacterium associated with the foodborne disease listeriosis in humans. Listeriosis affects thousands of people in the U.S. yearly, with neonates, elderly, pregnant women, and immunocompromised persons disproportionately affected. *Lm* has a high mortality rate in these populations (20 - 30%) with symptoms including septicemia, meningitis, encephalitis, and, in pregnant women, stillbirth and miscarriage. Due to the severity of the disease it causes, the U.S. has a zero-tolerance policy for *Lm* in ready-to-eat (RTE) food products. *Lm* is ubiquitous and often associated with food processing facilities, and the food processing environment itself is the main source of contamination in RTE products. Cleaning and sanitation are vital in reducing the presence of *Lm* in these areas. There are a variety of sanitizers available for use in the food industry. Among these are a widely used class of sanitizers called quaternary ammonium compounds (QUATs). Additionally, the U.S. Environmental Protection Agency (EPA) recently approved seven active ingredients for use in eco-friendly alternatives to traditional sanitizers. Despite these and other control methods, recalls and outbreaks due to *Lm* continue to occur. The objectives of this thesis were: (i) to determine the minimum bactericidal concentrations (MBCs) of two traditional (TR) sanitizers, including benzalkonium chloride (BAC) and a commercial QUAT (CQAC), and two eco-friendly (EF) sanitizers, including an alcohol-based (ALB) and a citric acid-based (CAB) sanitizer, against *Listeria* spp. (n=22) at temperatures ranging from 4 – 30^{o}C; (ii) to evaluate the behavior of *Lm* (n=8) in sub-lethal concentrations of these sanitizers, mimicking unintentional dilution that may occur in processing facilities; and (iii)

to assess the efficacy of sanitizers against *Lm* (n=5) attached to stainless steel (SS) and plastic at 4°C and 30°C for 24 h.

Minimum bactericidal concentrations (MBCs), based on the percentage of the manufacturer recommended concentration (% MRC), were significantly lower for TR sanitizers compared to those of EF sanitizers ($P < 0.001$; ANOVA). Temperature had a significant impact on MBCs of all sanitizers, with MBCs decreasing as temperature increased ($P < 0.05$; ANOVA). Similar growth behavior of isolates exposed to sub-lethal EF sanitizers was observed. In contrast, growth in sub-lethal concentrations of TR sanitizers revealed two isolates that were severely inhibited, confirming that differences in the ability of isolates to tolerate QUATs exist in the tested population.

Attachment to surfaces affected tolerance to sanitizers, with attached bacteria more tolerant than those in the planktonic state. All *Lm* isolates tested had survival rates above 50% when attached to SS and plastic at 30°C and treated with BAC and CAB at their MRC/ET. Incidence of inactivation for all sanitizers tested increased when both *Lm* attachment and sanitizer treatment occurred at 4°C. Differences in survival on two surface materials was also observed, with higher survival rates occurring with attachment on SS compared to plastic coupons. Scanning electron micrographs revealed greater population of bacteria on coupons when attached at 30°C compared to 4°C. Additionally, micrographs of plastic coupons showed they had a smoother surface compared to SS coupons. Notably, addition of a cleaning step, by scrubbing inoculated coupons (30°C, 24h) with cotton tips soaked in detergent, prior to treatment with sanitizers, decreased the incidence of survival on SS and plastic coupons treated with ALB and CAB; however, it did not significantly reduce survival of *Lm* on SS or plastic treated with QUATs.

Overall, the response of *Lm* isolates to sub-lethal concentrations varied the most with QUAT sanitizers, indicating that genetic variation between *Lm* strains may have a greater effect on the efficacy of these sanitizers. Attachment of *Lm* isolates on commonly used food contact surface materials greatly reduced the efficacy of all sanitizers tested. Food processing industry should consider cleaning and sanitizing more often than once every 24 h to reduce the risk of surface adherent *Lm* surviving cleaning and sanitation procedures. Collectively, these findings highlight important variables, such as sanitizer types, temperatures, and surfaces, that need to be carefully considered by food processing facilities when designing and implementing their cleaning and sanitation regimes.

Efficacy of Sanitizers at Controlling *Listeria* spp. and *Listeria monocytogenes* When Challenged with Variables Commonly Encountered in Food Processing Environments

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I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Cara Boucher, Author

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DEDICATION

To Paul, Cathy, Rachel, Ian, and Hazen Boucher, I love you all.

Chapter 1: Introduction

Listeria monocytogenes is a pathogenic bacterium responsible for causing the disease listeriosis in humans. It is the third leading cause of foodborne disease related deaths in the U.S., with pregnant women, neonates, elderly, and immunocompromised persons disproportionately affected (CDC, n.d.). Symptoms in these at-risk populations may include encephalitis, meningitis, septicemia and in pregnant women, fetal death (Farber and Peterkin, 1991). *Listeria monocytogenes* is ubiquitous in the natural environment, and it is often associated with food processing environments. It is resistant to high salinity, low pH, and low temperature conditions (Schuchat et al., 1991). In addition, its biofilm-forming abilities allow it to survive and persist in food processing environments for prolonged time (Ferreira et al., 2014). The processing environment itself presents the greatest contamination risk to ready-to-eat (RTE) foods (Farber and Peterkin, 1991).

With continual worker, product, and equipment movement in and out of processing areas, there are many ways *L. monocytogenes* can be introduced and spread through a facility. Frequent and efficient cleaning and sanitation regimes are considered essential to control environmental pathogens, such as *L. monocytogenes*, and to minimize their spread within processing environments, and from the environment to RTE foods. A wide variety of sanitizers have been approved by the Environmental Protection Agency (EPA) for application in food processing areas, in U.S. Additionally, the EPA has recently endorsed a selection of sanitizing compounds as eco-friendly as part of their Design for the Environment (DfE) program, increasing the visibility and availability of sanitizers with eco-friendly labels.

Along with cleaning and sanitation, new regulations as part of the Food Safety Modernization Act (FSMA) focus on a more holistic approach to food safety, including updated requirements for preventive controls, monitoring, product traceability, worker training and documentation. With the implementation ongoing, FSMA's intention is to shift focus from reacting to food safety events to preventing them from happening.

Despite current control measures and new regulations, recalls and outbreaks due to *L. monocytogenes* continue to occur. In 2018, over 70 recalls were issued by the Food and Drug Administration (FDA) and United States Department of Agriculture (USDA) due to contamination with this pathogen (FDA, 2019; USDA, n.d.). These events highlight the need to improve our efforts in controlling *L. monocytogenes*. To do this effectively, we need to better understand the

effect of conditions encountered in processing environments on the efficacy of sanitizers against *L. monocytogenes.* Additionally, as eco-friendly sanitizers continue to be integrated into the food industry, it is important to understand if the efficacy of these sanitizers is affected by conditions commonly encountered in food processing environments, such as variations in temperatures and bacterial attachment to food contact surfaces.

The purpose of this thesis, therefore, was to improve our understanding of how sanitizing compounds used in the food industry are affected by conditions encountered in these environments, such as exposures at various temperatures and their efficacy when bacteria are allowed to attach to common food contact surfaces.

1.1 Research objectives and hypotheses

This project had three research objectives, including:

1) Establish minimum bactericidal concentrations (MBCs) of traditional and eco-friendly sanitizers (n = 4) against *Listeria* spp. (n = 22) recovered from diverse food processing facilities and clinical settings when incubated at temperatures ranging from 4 – 30^{o}C.
   - Hypothesis 1.1: Isolate MBCs of traditional and eco-friendly sanitizers increase as temperature decreases.

2) Evaluate growth curves of a subset of *L. monocytogenes* isolates (n = 8) representing the most and least susceptible isolates, based on MBC data, when isolates are exposed to sub-lethal concentrations of sanitizers (n = 4).
   - Hypothesis 2.1: Isolates with lower MBCs adapt more slowly to sub-lethal concentrations of sanitizers.
   - Hypothesis 2.2: Food chain-derived *Listeria monocytogenes* have different adaptation rates (lag phase) in the presence of sub-lethal concentrations of QUATs.
   - Hypothesis 2.3: Food chain-derived *Listeria monocytogenes* have different growth rates in the presence of sub-lethal concentrations of QUATs.

3) Determine if sanitizers (n = 4) applied following manufacturer recommendations for concentration and exposure time are able to inactivate *L. monocytogenes* isolates (n = 5)

attached to common food contact surfaces when applied with and without a cleaning step prior to sanitizing.

- Hypothesis 3.1: Sanitizers (n = 4) applied following manufacturer recommendations for concentration and exposure time are able to inactivate *L. monocytogenes* isolates (n = 5) attached to plastic and stainless steel surfaces when a cleaning step is included prior to sanitizing.
- Hypothesis 3.2: Sanitizers (n = 4) applied following manufacturer recommendations for concentration and exposure time are able to inactivate *L. monocytogenes* isolates (n = 5) attached to plastic and stainless steel surfaces when applied without a cleaning step prior to sanitizing.
- Hypothesis 3.3: Temperature has an effect on bactericidal activity of sanitizers against attached (24 h) *L. monocytogenes*.

Chapter 2: Literature Review

2.1 Characteristics of the genus *Listeria*

Listeria is a genus of Gram-positive bacteria, currently consisting of 20 species (Leclercq et al., 2018). Discovered in 1924, *L. monocytogenes* was the first *Listeria* spp. described. It was identified by Murray et al. (1926), who determined a small Gram-positive bacillus was responsible for causing a septicemic disease in rabbits and guinea pigs in their laboratory (Murray et al., 1926). Over the subsequent 60 years, five additional *Listeria* spp. were discovered. The vast majority of species have been detected in the past decade thanks to developments in molecular techniques, particularly whole genome sequencing (Orsi and Wiedmann, 2016).

The *Listeria* genus is split into two groups based on the species relatedness to *L. monocytogenes*. *Listeria sensu stricto* is a monophyletic group containing the older *Listeria* species (*L. monocytogenes, L. innocua, L. ivanovii, L. seeligeri,* and *L. welshimeri*), along with a more recently discovered species (*L. marthii*). *Listeria sensu lato* contains the remaining 12 species (Table 2.1) (Orsi and Wiedmann, 2016). Although all *Listeria* spp. are phenotypically similar, some differences exist. For example, all species in *Listeria sensu stricto* are motile at 30^{o}C, while within *Listeria sensu lato* only *L. grayi* is motile. Also, the only hemolytic species are found within *Listeria* sensu *stricto* (*L. monocytogenes, L. ivanovii, L. seeligeri*) (Orsi and Wiedmann, 2016).

All *Listeria* spp. are non-sporeforming, non-capsulated, small rods. They are psychrotrophic and capable of growing at refrigeration temperatures (e.g. 4^{o}C) (Farber and Peterkin, 1991). *Listeria* spp. are primarily saprophytic and are widespread in the environment (Fenlon, 1999). Only two species within the *Listeria* genus are pathogenic. *Listeria ivanovii* and *L. monocytogenes* have both been reported to cause the disease listeriosis in humans and ruminant animals. However, human cases involving *L. ivanovii* are very rare and it is not considered to be a major public health concern (Guillet et al., 2010). Conversely, *L. monocytogenes* is recognized as one of the deadliest foodborne pathogens, causing the third most foodborne disease-related deaths in the U.S., particularly affecting vulnerable populations, such as elderly, immunocompromised, pregnant women, fetuses, and newborns (Scallan et al., 2011).

Table 2.1. List of all *Listeria* spp. discovered to date and their grouping into *Listeria sensu stricto* or *Listeria sensu lato.*

Species	Group	Year Discovered	References
L. monocytogenes	*sensu stricto*	1924	Murray et al., 1926; Pirie, 1940
L. innocua	*sensu stricto*	1977	Seeliger, 1981
L. welshimeri	*sensu stricto*	N/A - 1983*	Rocourt and Grimont, 1983
L. seeligeri	*sensu stricto*	N/A - 1983*	Rocourt and Grimont, 1983
L. ivanovii	*sensu stricto*	N/A - 1984*	Seeliger et al., 1984
L. grayi	*sensu lato*	N/A - 1992*	Rocourt et al., 1992
L. rocourtiae	*sensu lato*	N/A - 2002	Leclercq et al., 2010
L. marthii	*sensu stricto*	N/A - 2010*	Graves et al., 2010
L. fleischmannii	*sensu lato*	2006	Bertsch et al., 2013
L. weihenstephanensis	*sensu lato*	N/A - 2013*	Halter et al., 2013
L. floridensis	*sensu lato*	N/A - 2014*	den Bakker et al., 2014
L. aquatica	*sensu lato*	N/A - 2014*	den Bakker et al., 2014
L. cornellensis	*sensu lato*	N/A - 2014*	den Bakker et al., 2014
L. riparia	*sensu lato*	N/A - 2014*	den Bakker et al., 2014
L. gardensis	*sensu lato*	N/A - 2014*	den Bakker et al., 2014
L. booriae	*sensu lato*	N/A - 2015*	Weller et al., 2015
L. newyorkensis	*sensu lato*	N/A - 2015*	Weller et al., 2015
L. costaricensis	*sensu lato*	2015	Nunez-Montero et al., 2018
L. goaensis	*sensu lato*	N/A - 2018*	Doijad et al., 2018
L. thailandensis	*sensu lato*	2015	Leclercq et al., 2018

*Year species was reported.

2.1.1 *L. monocytogenes* and listeriosis disease

The propensity of *L. monocytogenes* to cause infections in humans makes it unique among *Listeria* spp. While it primarily exists as a saprophyte, *L. monocytogenes* is also a successful human pathogen due to its distinctive set of virulence genes. During its infection cycle, *L. monocytogenes* enters both professional and non-professional phagocytic cells, chiefly macrophages and epithelial cells respectively, in order to replicate (Camejo et al., 2011). In most cases, *L. monocytogenes* enters the host through contaminated food and infection begins at epithelial cells in the small intestine (Portnoy et al., 2002). It attaches to epithelial cells and induces phagocytosis via internalin

proteins (InlA and InlB) (Dramsi et al., 1995; Gaillard et al., 1991). Once internalized, *L. monocytogenes* uses a pore-forming toxin, listeriolysin O (LLO), assisted by two phospholipases (PlcA and PlcB) to escape its vacuole (Gedde et al., 2000; Goldfine and Knob, 1992). Now in the cytoplasm, the bacteria can replicate, stealing nutrients from the host cell. Prior to entering the host, *L. monocytogenes* has peritrichous flagella, used to react to stimuli in the environment and to increase host cell invasion (O'Neil and Marquis, 2006). However, once inside a host cell, flagellar genes are down-regulated and *L. monocytogenes* switches to actin polymerization for movement (Grundling et al., 2004). Using the protein ActA, *L. monocytogenes* recruits host cell actin and other structural proteins to navigate through the cell. This actin polymerization aids in cell-to-cell movement by pushing against the host cell membrane and forming a protrusion, which is taken up by the next cell (O'Neil and Marquis, 2006; Portnoy et al., 2002). Using LLO, the bacteria escape from the double-membrane vacuole into the cytoplasm of the new host cell, thus spreading the infection.

In healthy adults, *L. monocytogenes* may cause gastroenteritis, but the infection does not typically progress past this point. However, in susceptible populations (i.e. children, elderly, pregnant women, and immunocompromised persons), the lack of a fully functioning immune system allows the infection to persist and spread (Farber and Peterkin, 1991). In fact, *L. monocytogenes* is capable of crossing several important protective barriers in the human body, including the intestinal, blood-brain, and feto-placental barriers (Lecuit, 2005). In more severe infections, *L. monocytogenes* will enter the bloodstream and may colonize the liver, brain, and placenta. These systemic infections can result in sepsis, meningitis, death, and stillbirth or spontaneous abortion in pregnant women (Schuchat et al., 1991). Mortality rates from these systemic infections often reach 20-30% (Camejo et al., 2011).

Whether or not symptoms appear is highly dependent upon the health of the host, as well as the number of invading bacteria and the virulence of individual strains. The exact infectious dose has not been determined, but analysis of food samples responsible for outbreaks suggest that it is high (around 10^6 CFU/ml) (Vazquez-Boland et al., 2001). However, the dose drops significantly in susceptible populations. Foods with *L. monocytogenes* populations as low as 100 CFU/ml have been found to cause disease in humans (CDC, 2015; Farber and Peterkin, 1991).

In addition to sophisticated mechanisms that *L. monocytogenes* use to cause human disease, this microorganism possesses a number of characteristics that allow it to survive a variety of harsh

environmental conditions. It is resistant to acidic and high salinity environments; it can grow at refrigeration temperatures; and it is able to form biofilms. These characteristics allow *L. monocytogenes* to persist and even thrive in various food processing environments and food matrices (Swaminathan and Gerner-Smidt, 2007). Combining its hardy nature together with the devastating disease it causes, leads to *L. monocytogenes* being a significant concern to the food industry.

2.2 Outbreaks and recalls

The first foodborne disease outbreak linked to *L. monocytogenes* occurred in Nova Scotia, Canada in 1981. It was associated with contaminated coleslaw, leading to 41 hospitalizations. Mortality rates of 27% and 33% were reported in perinatal and adult cases, respectively (Schlech et al., 1983). It is likely that *L. monocytogenes* had been responsible for previous foodborne outbreaks, but this was the first time the connection between contaminated foods and illnesses was established. A series of biochemical tests, including Gram-stain, Vogues-Proskauer, catalase, motility and β-hemolysis was used to confirm *L. monocytogenes* as the etiological agent in this outbreak. Three decades later, advances in molecular techniques have provided new, quicker and more precise detection methods for foodborne pathogens, and led to improvements in tracking and identifying foodborne contamination events. Collecting information about what products are affected and the organisms responsible help to inform and update industry practices (Dewey-Mattia et al., 2018). For decades, pulsed-filed gel electrophoresis (PFGE) method was considered the "gold standard" for isolate fingerprinting during outbreak investigations. In 2013, the Centers for Disease Control and Prevention (CDC) implemented the use of the whole genome sequencing (WGS) in parallel with PFGE, during listeriosis outbreak investigations. The use of WGS not only increased the ability to identify identical strains, improving linking of cases of listeriosis to outbreaks and tracking *L. monocytogenes* incidence in foods, but it also provided information about the virulence and stress resistance of individual isolates (CDC, 2016a). In 2019, WGS became the new PulseNet gold standard for identifying foodborne pathogens (CDC, 2019a).

In North America, *L. monocytogenes* has been found to be responsible for 16 outbreaks since 2011 (Table 2.2). One of the more prominent listeriosis outbreaks in recent years was caused by cantaloupes in 2011. This outbreak led to 147 confirmed cases of listeriosis, with 143 hospitalizations and 33 deaths across 28 states (CDC, 2012a). Through the environmental

assessment, the Food and Drug Administration (FDA) learned that trucks being used to transport melons to nearby cattle operations may have played a role in introducing *L. monocytogenes* to the farm. Additionally, water from the packing facility and various farm equipment tested positive for *L. monocytogenes* (McCollum et al., 2013). In fact, it is now well established that water, soil, and decaying vegetation are important environmental reservoirs of *L. monocytogenes* (Fenlon, 1999). Additionally, animals can be carriers of *L. monocytogenes* and shed the pathogen through their feces, aiding in its further dispersion in agricultural settings (Nightingale et al., 2004). Once introduced to a food processing facility, *L. monocytogenes* can quickly spread through worker, equipment, and product movement (El-Shenawy, 1998; Kovacevic et al., 2012). As such, *L. monocytogenes* is considered an environmental pathogen, with food processing facilities posing the greatest re-contamination risk to food products (Farber and Peterkin, 1991; Tompkin, 2002).

Table 2.2. List of foodborne outbreaks of listeriosis in the United States since 2011.

Implicated Food	Year	# of Cases	# of Deaths (Mortality Rate)	Reference
Cantaloupe	2011	147	33 (22%)	(CDC, 2012a)
Cheese	2012	22	4 (18%)	(CDC, 2012b)
Cheese	2013	6	1 (16.5%)	(CDC, 2013)
Dairy products	2014	8	1 (12.5%)	(CDC, 2014a)
Cheese	2014	5	1 (20%)	(CDC, 2014b)
Bean sprouts	2014	5	2 (40%)	(CDC, 2015a)
Caramel apples	2014	35	7 (20%)	(CDC, 2015b)
Ice cream	2015	10	3 (30%)	(CDC, 2015c)
Cheese	2015	30	3 (10%)	(CDC, 2015d)
Packaged salad	2016	19	1 (5%)	(CDC, 2016b)
Raw milk	2016	2	1 (50%)	(CDC, 2016c)
Frozen vegetables	2016	9	3 (33%)	(CDC, 2016d)
Raw milk cheese	2017	8	2 (25%)	(CDC, 2017)
Deli ham	2018	4	1 (25%)	(CDC, 2018)
Pork products	2018	4	0 (0%)	(CDC, 2019b)
Deli-sliced meats and cheeses	2019	8	1 (12.5%)	(CDC, 2019c)

On average, *L. monocytogenes* causes 1,600 human illnesses every year in the U.S. Of these, around 1,500 cases require hospitalization and 260 people die from the disease (CDC, n.d.). Compared to other foodborne pathogens, such as *Salmonella*, which is responsible for an average of 1.2 million illnesses every year, *L. monocytogenes* infections are rare. Despite this, *L. monocytogenes* causes the third most foodborne disease related deaths in the U.S. (Scallan et al., 2011). With approximately 94% of confirmed listeriosis cases requiring hospitalization, the medical care costs in the U.S. associated with this pathogen are exorbitant. In 2013, the USDA's Economic Research Service estimated that medical care costs due to *L. monocytogenes* during that year were over $2.8 billion (USDA, 2014).

In addition to medical costs, recalls due to contaminated products pose a substantial economic burden for food producers. Due to the severity of the illness, the U.S. has a zero-tolerance policy for *L. monocytogenes* in ready-to-eat (RTE) foods. If it is found in RTE foods, all potentially affected products must be recalled. In 2018, the FDA issued 52 recalls due to *L. monocytogenes* contamination in products ranging from cream cheese to frozen vegetables (FDA, 2019). The United States Department of Agriculture (USDA) also recalled over 4 million pounds of meat, poultry, and produce products, associated with 21 different recalls due to *L. monocytogenes* contamination (USDA, n.d.). In addition to the immediate financial impact caused by recalls, companies often experience a subsequent drop in revenue due to consumer avoidance of their products. Considering the risk that *L. monocytogenes* poses to public health and the economic burden on food producers and processors, a multi-stage approach is imperative to control this pathogen in food industry.

2.3 Methods of monitoring and controlling for *L. monocytogenes*

Listeria monocytogenes has several attributes that make it difficult to control in processing environments. It is facultatively anaerobic, resistant to high salinity and low pH environments, and it can grow at refrigeration temperatures (Swaminathan and Gerner-Smidt, 2007). It can also form biofilms, which contribute to its persistence in food processing environments for years (Vogel et al., 2010). Preventing the establishment of *L. monocytogenes* in a processing facility is a key step towards decreasing the risk of food contamination.

The latest U.S. legislation regarding food safety, the Food Safety Modernization Act (FSMA), emphasizes the prevention of food safety contamination events from occurring. Increased

focus has been placed on transparency amongst producers and buyers, and improved training, monitoring, recordkeeping, and traceability. The two main sections of this law, the Produce Safety Rule (PSR) and the Preventive Controls for Human Food (PCHF), outline new requirements for growers and processors, respectively, to facilitate the production of safer food. As the last legislation regarding food safety was the Food Drug and Cosmetic Act of 1938, FSMA was desperately needed to update industry practices to reflect current food safety knowledge.

As part of the PCHF, environmental monitoring programs (EMP) are required for facilities producing RTE foods that have identified the potential for recontamination of their product prior to packaging (21 C.F.R. § 117 (2018)). Similarly, the USDA Food Safety and Inspection Service (FSIS) has additional requirements for facilities producing RTE meat and poultry products that are exposed to the processing environment immediately prior to packaging. While sampling of the processing environment is recommended for all facilities, it is required for food contact surfaces in facilities that rely on the use of an antimicrobial agent in the food to suppress growth of *L. monocytogenes*, facilities that use sanitation alone to control *L. monocytogenes,* and facilities that are producing deli meat or hot dogs (FSIS, 2014).

Environmental monitoring consists of sampling the processing area and testing for environmental pathogens, most commonly *L. monocytogenes* and *Salmonella* spp. Facilities may choose to test finished products and food contact surfaces. If *L. monocytogenes* is found in the food or on food contact surfaces, facilities are required to issue a recall of the implicated product (FDA, 2017). Often, facilities choose to test for *L. monocytogenes,* or general *Listeria* spp. without further speciation, on non-food contact surfaces only. These practices have been useful in discovering potential harborage sites of environmental pathogens within a facility, without requiring automatic recall if *L. monocytogenes* is found. When positive results (i.e. *L. monocytogenes* or *Listeria* spp., if not speciating) are encountered, processors are expected to clean, sanitize, and retest the implicated area, ensuring that a contamination threat is effectively removed from their facility. It is also recommended to record positive results against the blueprint of the facility to aid in hypothesizing microbial spread through the facility, and inform personnel of practices that may increase the risk of food contamination. Since contamination of RTE foods with *L. monocytogenes* is of high concern and environmental monitoring programs are an early warning system for food contaminants, implementing EMP is a recommended practice even in those facilities where laws do not require them.

In food, *L. monocytogenes* can be eliminated through a processing kill step, such as pasteurization or cooking, or controlled through altering qualities of the food so that it will not support the growth of bacteria. Acidified foods and low moisture foods, defined as foods with pH less than 4.6 and water activity (a_w) less than 0.85, respectively, prevent bacterial growth but may not help to decrease the number of bacteria already present (FDA, 2018; Sanchez-Maldonado et al., 2018). For products with a processing kill step, preventing recontamination after the kill step is essential to maintain the safety of the product. However, microbial inhibition via formulation and processing kill step methods such as these are limited to products whose quality will not be negatively affected by these techniques.

Alternatively, there are post-packaging control methods that may be employed, including high pressure processing (HPP) and gas-phase treatments. In HPP, products are subjected to pressures as high as 600 MPa in order to kill vegetative spoilage or pathogenic microorganisms that may be present (Smelt, 1998). As *L. monocytogenes* is a nonsporeformer, HPP is an effective control method. However, HPP may negatively affect quality of some foods, such as raw meat and soft or spongy food products (Considine et al., 2008). In addition, HPP machines are expensive, which may exclude smaller facilities from using this technology. An alternative is to fumigate packaged foods with antimicrobial gases, such as ozone and chlorine dioxide, although more research is needed to assess effectiveness of these methods with different packaging materials (Murray et al., 2017).

Cleaning and sanitation of the processing facility is considered a key food safety component, and if done adequately it provides a cost-efficient and effective control method for environmental pathogens. A wide range of sanitizing compounds is available for use in food processing environments. Quaternary ammonium compounds (QUATs) represent a large class of sanitizers that have been widely used in the food industry for over 50 years (Gerba, 2015). These compounds are surface active. They contain both hydrophobic and hydrophilic regions that allow them to adsorb and insert into cytoplasmic membranes of microorganisms. Bacterial death results from the leakage of cellular components and degradation of nucleic acids and proteins (McDonnell and Russell, 1999). Unlike antibiotics, which are typically designed to target very specific processes, the mode of action for sanitizers are less specific and can be hard to elucidate (Maris, 1995). Sanitizers often cause membrane damage, leading to the leakage of intracellular components and subsequent destruction of proteins and nucleic acids by the sanitizer. This is the

general method of attack used by many sanitizers, including alcohol-, peracetic acid-, hydrogen peroxide- and chlorine-based compounds (CDC, 2008; McDonnell and Russell, 1999).

2.4 Antimicrobial tolerance in *L. monocytogenes*

Antimicrobials are compounds that suppress or kill microorganisms. There are several types of bacteriostatic and bactericidal compounds for bacterial suppression and inactivation, respectively, including preservatives, antibiotics, and sanitizers. Sanitizers are commonly used in the food industry, as well as in industrial, medical, and household settings. Similar to increased use of antibiotics and the corresponding evolution of antibiotic resistant bacteria, there is a growing concern over the widespread use of sanitizers and the increasing sanitizer tolerance of pathogenic bacteria (Morente et al., 2013; Romanova et al., 2006).

Antimicrobial resistance can be obtained through genetic mutation or acquired through the uptake of exogenous resistance genes from other bacteria (Walsh et al., 2001). This may not necessitate that bacteria share the same species or even the same genus. Listeriae, enterococci, and *Staphylococcus aureus* have shown on several occasions to share plasmids and transposons encoding for antimicrobial resistance (Charpentier and Courvalin, 1999). Additionally, naturally occurring stress response genes, activated by stressors such as extreme temperature and presence of antimicrobials, can aid in the survival of the bacteria. Often, this includes altering the physiology of normal cell functions (van Shaik and Abee, 2005). When combined, acquired resistance mechanisms can allow bacteria to endure harsh conditions.

2.4.1 Antibiotic resistance and sanitizer tolerance in *L. monocytogenes*

The first description of antibiotic resistant *L. monocytogenes* dates back to 1988, when Poyart-Salmeron et al. (1990) discovered a clinical *L. monocytogenes* strain with resistance to erythromycin, streptomycin, chloramphenicol, and tetracycline. Since then, research in this area revealed multiple strains with resistance to one or more antibiotics (Charpentier and Courvalin, 1999). This was surprising, as *L. monocytogenes* was long believed to be susceptible to antibiotics effective against Gram-positive bacteria (Poyart-Salmeron et al., 1990).

Listeria monocytogenes is naturally resistant to cephalosporins, fosfomycin, and early generations of fluoroquinolones (Hof et al., 1997; Morvan et al., 2010; Troxler et al., 2000). Both,

food and clinical *L. monocytogenes* isolates with acquired resistance to a variety of antibiotics have also been reported. This includes isolates with tetracycline and sulfonamide resistance and occasional resistance to erythromycin, trimethoprim, gentamicin, clindamycin, chloramphenicol, kanamycin, rifampicin, and streptomycin (Charpentier et al., 1995; Facinelli, 1991; Morvan et al., 2010; Poyart-Salmeron et al., 1990; Troxler et al., 2000; Tsakris et al., 1997; Zhang et al., 2007). Resistance to these antibiotics has been acquired mostly through the uptake of self-transferable plasmids or direct conjugative transfer of plasmids and transposons, often associated with enterococci, streptococci or *Staphylococcus aureus* (Charpentier and Courvalin, 1999). Resistance to relevant listeriosis drug therapies has been rare, but occasional reports of resistance warrant caution when prescribing treatment. Typically, treatment of listeriosis uses a combination of a β-lactam antibiotic, such as penicillin or ampicillin, and an aminoglycoside, most often gentamicin. In case of β-lactam allergy, alternative therapies using erythromycin, tetracycline, or most commonly, trimethoprim may be employed (Charpentier et al., 1995; Hof, 1991; Zhang et al., 2007).

Overall, the mechanisms behind antibiotic resistance in bacteria involve one or more of the following: (i) production of enzymes that detoxify the antibiotic; (ii) modification of the antibiotic's target; and (iii) active efflux of the antibiotic from the bacteria (Leclercq and Courvalin, 2002; Speer et al., 1992). Antibiotic efflux is facilitated by active transporters located in the bacterial membrane that pump the antibiotic out of the cell. These transporters are referred to as efflux pumps. Efflux pumps are highly varied; with pumps recognizing single or multiple substrates, and with structures ranging from single peptides to multiple component systems described (Morente et al., 2013; Putman et al., 2000). There are five main classes of efflux pumps, including the major facilitator (MF) superfamily, ATP-binding cassette (ABC) family, resistance-nodulation-division (RND) family, small multidrug resistance (SMR) family, and multidrug and toxic compound extrusion (MATE) family (Poole, 2005). These classes represent a collection of efflux pumps capable of providing protection from a wide array of therapeutically relevant antibiotics, as well as from heavy metals, preservatives, and sanitizers (Morente et al., 2013; Poole, 2005).

Several antimicrobial efflux pumps have been documented in *L. monocytogenes* (Elhanafi et al., 2010; Godreuil et al., 2003; Kovacevic et al., 2016; Mata et al., 2000). From the MF superfamily, *lde* encodes an efflux pump that provides resistance to fluoroquinolones, as well as

limited protection against acridine orange and ethidium bromide (Godreuil et al., 2003). Similar to most other efflux pumps that belong to the MF superfamily, Lde uses proton motive force to remove deleterious compounds from the cell (Godreuil et al., 2003; Quistgaard et al., 2016). MdrL, another MF superfamily efflux pump, works against several antibiotics (macrolides and cefotaxime), as well as ethidium bromide and heavy metals, including zinc, cobalt, and chromium (Mata et al., 2000). Romanova et al. (2006) found that treatment of *L. monocytogenes* with the QUAT, benzalkonium chloride (BAC), in the presence of reserpine, an efflux pump inhibitor, increased isolate sensitivity to BAC. Subsequent gene expression work revealed that *mdrL* expression increased during treatment with BAC, suggesting that MdrL also provides protection from BAC. This finding aligns with previous reports of a general increase in tolerance attributed to non-specific or multidrug efflux pumps (Paulsen and Skurray, 1996; Romanova et al., 2006). Similarly, efflux pumps EmrE and BcrABC also contribute to QUAT tolerance in *L. monocytogenes.* These efflux pumps have been found in both clinical isolates and those collected from food and food processing environments (Dutta et al., 2013; Hingston et al., 2017b; Kovacevic et al., 2013). While tolerance levels in these isolates are still well below manufacturer recommended concentrations for QUATs, if improper usage of sanitizers in processing facilities leads to exposure of *L. monocytogenes* to sublethal sanitizer concentrations it may result in stress-adapted strains that are better able to survive and persist in food processing environments (Hingston et al., 2017a; Martinez-Suarez et al., 2016).

In addition to antimicrobial resistance genes, increased tolerance to sanitizers and other antimicrobials can be attained through morphological changes. Alterations in fatty acid composition and increased peptidoglycan cross-linking have been suggested to aid in increasing tolerance to BAC (Romanova et al., 2006; To et al., 2002). Exposure to stress, such as sublethal sanitizer concentrations, activates a network of stress response mechanisms in *L. monocytogenes*, including physiological changes and cell membrane modifications that may impart antimicrobial resistance and increased chance of survival (van Schaik and Tjakko, 2005).

2.4.2 Stress response in *L. monocytogenes*

Similar to other Gram-positive bacteria, stress response mechanisms in *L. monocytogenes* are mediated by sigma-B (σ^B) factor, a protein that promotes the transcription of a set of genes that

protect the bacterium in adverse conditions (van Schaik and Tjakko, 2005). Food processing environments present a wide array of stress sources, including low temperatures in coolers, decreased availability of nutrients, and presence of sanitizers. Sigma-B in *L. monocytogenes* activates in response to these stressors (i.e. extreme temperatures, starvation, and antimicrobials). Its activation is also triggered by oxidative stress, low or high pH, and high osmolarity (Hecker et al., 2007; Kazmierczak et al., 2003; van Schaik and Tjakko, 2005). There is an estimate of 150 σ^B dependent genes with function in stress response, transport, metabolism, and virulence, as well as many that have no function yet described (Kazmierczak et al., 2003).

The σ^B regulon also helps *L. monocytogenes* grow in cold temperatures (Annous et al., 1997). Low temperatures cause cellular membranes to become rigid, inhibiting the exchange of nutrients and waste products. In order to grow at low temperatures, the membrane fluidity needs to be maintained (Annous et al., 1997). In *L.* monocytogenes, this is accomplished through two routes. The first is through the alteration of fatty acid composition in the membrane to maintain fluidity; a phenomenon referred to as homeoviscous adaptation (Annous et al., 1997; Sinensky, 1974). The second is through the accumulation of compatible solutes, such as glycine betaine, carnitine, and proline (Becker et al., 2000; Ko et al., 1994). While the exact mechanisms behind cryoprotection afforded by these solutes are yet to be elucidated, σ^B regulon has been shown to control gene expression for several transporters of these solutes (e.g. *opu* transporters) (Becker et al., 2000; Kazmierczak et al., 2003). This ability to grow at cold temperatures is highly problematic for the food industry, as any *L. monocytogenes* that survive following food processing and handling may be able to grow to harmful levels during storage in coolers and refrigerators (Farber and Peterkin, 1991).

Collectively, stress response genes aid the survival of *L. monocytogenes* in extreme temperatures, low pH (4.1 – 9.6) and high salinity environments (10-20%), as well as low nutrient and dry conditions (Abeysundara et al., 2019; Hecker et al., 2007; Kazmierczak et al., 2003; van Schaik and Tjakko, 2005). Combined with the abilities of *L. monocytogenes* to form biofilms and acquire antimicrobial resistance genes, these properties greatly contribute to the survival of *L. monocytogenes* in harsh conditions in natural environments, as well as conditions encountered in food processing environments and a wide variety of food matrices.

Chapter 3: Investigation of the Minimum Bactericidal Concentrations and Growth Behavior in the Presence of Sub-Lethal Concentrations of Traditional and Eco-Friendly Sanitizers against *Listeria* spp. Recovered from Diverse Food Processing Facilities

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Abstract

Growth of *Listeria monocytogenes* (*Lm)* in cold temperatures and its tolerance of antimicrobials can promote its survival and persistence in food processing environments (FPE). With food industry relying on cleaning and sanitation to control *Lm* in FPE, it is important to understand the effect of conditions encountered in FPE on the efficacy of antimicrobials used in food industry. The minimum bactericidal concentrations (MBCs) of four sanitizers (2 QUATs, 2 with eco-friendly [EF] ingredients) against 22 *Listeria* spp. isolates were assessed at 4-30ºC. A subset of *Lm* isolates (n=8) were also exposed to sub-lethal concentrations of the sanitizers to assess differences in growth behavior. All sanitizers were effective at manufacturer recommended concentrations. MBCs increased as temperature decreased. MBCs of ALB, CQAC, and CAB were significantly lower at 30ºC compared to 4ºC, and at 23ºC compared to 4ºC for BAC (P<0.05). Temperature had the least effect on CQAC. Two *Lm* were unable to grow in sub-lethal QUAT concentrations tested. All isolates grew similarly in sub-lethal EF sanitizers based on the lag phase duration, maximum growth rate, and maximum optical density. This work highlights variables encountered in FPE and FPE isolates that affect sanitizer efficacy and should be considered by food industry.

Keywords:
Listeria monocytogenes, sanitizers, eco-friendly, cold temperature, MBCs, sub-lethal

Highlights:

- Sanitizers inactivated *Listeria* spp. at manufacturer recommended concentrations
- Higher MBCs were needed to inactivate *Listeria* at lower temperatures
- MBCs of commercial QUAT sanitizer were least affected by temperature variation
- Isolates had similar growth at sublethal concentrations of eco-friendly sanitizers

3.1 Introduction

Listeria monocytogenes is a foodborne pathogen responsible for causing the disease listeriosis in humans. In susceptible populations, including children, elderly, pregnant women and immunocompromised persons, the disease can be severe and lead to septicemia, encephalitis, meningitis, and death (Schuchat et al., 1991). Due to its resistance to high salinity and low pH environments, ability to form biofilms and grow at refrigeration temperatures (4^{o}C) (Swaminathan and Gerner-Smidt, 2007), *L. monocytogenes* is one of the most problematic pathogens in the food chain. These attributes make it well-suited to surviving conditions found in food processing environments, where, if given the right conditions, it has been shown to persist for years (Vogel et al., 2010). As such, the food processing environment presents the highest risk of contamination to ready-to-eat (RTE) food products (Farber and Peterkin, 1991).

The food industry relies heavily on cleaning and sanitation regimes to prevent *L. monocytogenes* from becoming established in niches and harborage sites in the food processing environment. A wide selection of sanitizers is available for food processors to use on food contact (FCS) or non-food contact surfaces (NFCS). Due to their noncorrosive nature and effectiveness against a wide variety of bacteria, quaternary ammonium compounds (QUATs) are commonly used on both FCS and NFCS (Gerba, 2015; McDonnell and Russell, 1999; Tompkin, 2002). Other sanitizer classes, including chlorine releasing agents (CRAs), iodophors, peroxygens (i.e. peracetic acid, hydrogen peroxide), and alcohols, are also available for use in the food industry (CDC, 2008; McDonnell and Russell, 1999). Recently, the Environmental Protection Agency (EPA) approved seven antimicrobial compounds for use as active ingredients in "eco-friendly" sanitizers as part of their Design for the Environment (DfE) program. The approved compounds were chosen for their low toxicity and decreased potential to detrimentally effect the environment (EPA, n.d.).

With the extensive use of sanitizers in medical and food industries, and potential for bacterial exposures to sub-lethal concentrations, the development of increased tolerance and eventual resistance to these compounds is a significant concern. While no bacteria have been found to have complete resistance to any sanitizers, strains with increased tolerance levels have been recovered from food processing environments (Mullapudi et al., 2008). Strains of *L. monocytogenes* possessing a variety of efflux pumps, which serve as active transporters to facilitate the removal of bactericidal compounds from the bacteria, have been described (Elhanafi et al., 2010; Kovacevic et al., 2016; Romanova et al., 2006). Increased tolerance to antimicrobials

is often associated with the possession of one or more efflux pumps, though mechanisms controlling membrane fluidity and stress response have also been reported to play a role in antimicrobial tolerance (Romanova et al., 2006; To et al., 2002). Repeated exposure to sub-lethal concentrations may also lead to increased tolerance of *L. monocytogenes* to the antimicrobial, as well as in some cases to co-selection and cross-resistance (Katharios-Lanwermeyer et al., 2012; Kovacevic et al., 2013). Yu et al. (2018) found that repeated exposure to QUAT benzalkonium chloride (BAC), not only resulted in increased tolerance to BAC, but also decreased the susceptibility of *L. monocytogenes* to other bactericidal compounds including cefotaxime, cephalothin, ciprofloxacin, and ethidium bromide. In processing facilities, the unintentional dilution of sanitizers, which may occur from improper sanitizer preparation or mixing with standing water within the facility, can result in areas treated with sub-lethal sanitizer concentrations. Presence of *Listeria* spp. in these environments may select for stress-adapted progeny. Furthermore, reports that exposure to cold temperatures can lead to *L. monocytogenes* isolates becoming more tolerant to osmotic stress, basic conditions, and a variety of antibiotics (Abeysundara et al., 2019; Al-Nabulsi et al., 2015) are particularly concerning. As RTE food products are often stored at low temperatures, this condition may further exacerbate *L. monocytogenes* tolerance to antimicrobials.

There is a need to better understand how *L. monocytogenes* behave in the presence of sub-lethal sanitizer concentrations. With incorporation of eco-friendly ingredients into sanitizing protocols, there is also a need to better understand the efficacy of these sanitizers against *L. monocytogenes* originating from food processing environments, and the effect of variables such as temperature on minimum bactericidal concentrations (MBCs). The goal of this study was twofold: (i) to determine MBCs of two QUATS and two sanitizers with eco-friendly ingredients, at temperatures ranging from 4°C to 30°C against a diverse set of *Listeria* spp. isolates, and (ii) to

investigate growth potential of *L. monocytogenes* isolates when exposed to sub-lethal concentrations of the sanitizers.

3.2 Materials and methods

3.2.1 Bacterial isolates

A total of 22 *Listeria* spp. isolates were included in this study (Table 3.1), originating from various sources. Six isolates possessed known genetic elements that confer increased tolerance to quaternary ammonium compounds (e.g. *bcrABC*, *emrE*). Isolates were stored in a trypticase soy broth (TSB; Acumedia, Neogen, Lansing, MI) and 50% glycerol solution at -80°C. Isolates were resuscitated on trypticase soy agar (TSA; Acumedia, Neogen) supplemented with 5% defibrinated sheep blood (BAP; Hardy Diagnostics, Santa Maria, CA) with incubation at 37°C for 24 h. BAP plates were stored at 4°C and used for a maximum of two weeks.

3.2.2 Selection and preparation of antimicrobials

A total of four antimicrobials were used in this study, including two QUATS, referred to as traditional sanitizers, and two with ingredients recognized on EPA's DfE list, referred to as "eco-friendly" sanitizers (Table 3.2). The traditional sanitizers included a QUAT, benzalkonium chloride (BAC; Acros Organics, Geels, Belgium), and a commercially available QUAT Lysol® (CQAC; Professional Lysol No Rinse Sanitizer, Lysol, Parsippany, NJ). The eco-friendly sanitizers included an alcohol-based (ALB; Purell™ Food Service Surface Sanitizer, Purell, Akron, OH) and a citric acid-based sanitizer (CAB; PRO-SAN®L, Microcide Inc., Mountain View, CA), with their active ingredients included in the EPA's DfE list of approved eco-friendly compounds.

Antimicrobials were used as-is or prepared according to the manufacturer recommendations and stored at 4°C for up to one week. For QUAT sanitizers, QUAT

concentrations were measured using QUAT test strips (MQuant Quaternary Ammonium Compound test strips, MilliporeSigma, Burlington, MA).

3.2.3 Determination of minimum bactericidal concentration (MBC)

Minimum bactericidal concentrations (MBCs) were determined using the broth microdilution method previously described by Kovacevic et al. (2013). Single colonies were transferred from BAP plates into 5 ml of TSB, followed by incubation at 37ºC for 16±2 h with shaking (150 rpm; Thermo Scientific, MaxQ4000, Waltham, MA). Cultures were diluted to approximately 7 log CFU/ml in 0.1% peptone water (PW; Acumedia). Inoculum was confirmed by enumeration using the track dilution method described by Jett et al. (1997). Briefly, samples were serially diluted 1:10 in PW, plated side by side on TSA plates, and incubated at 37ºC for 24 h.

Table 3.1 *Listeria* spp. isolates (n=22) included in this experiment. Isolates were clinical or recovered from food or various food processing environments (PE) in Canada and U.S.

Isolate ID	Origin	Serotype	S-AMR[1] Genes	Reference
L. innocua				
ME19-1	Meat PE	N/A	N/A	Kovacevic et. al (2012a)
WRLP-357	Produce PE	N/A	N/A	Jorgensen (2019)
L. monocytogenes				
Scott A	Clinical	4b	N/A	A. Yousef[2]
OSY-428*[3]	Meat PE	N/A	N/A	A. Yousef[2]
Ohio	Dairy PE	4b	N/A	A. Yousef[2]
ATCC® 19116	Meat PE	4c	N/A	
81-0861	Food	4b	N/A	Knabel et. al (2012)
08-5578*	Clinical	1/2a	*emrE*	Gilmour et. al (2010)
08-5578_Δ1862*	Clinical	1/2a	*emrE* deleted	Kovacevic et. al (2016)
FE7-1*	Fish PE	1/2c	*bcrABC*	Kovacevic et. al (2012a)
FE10-1*	Fish PE	3a	*bcrABC*	Kovacevic et. al (2012a)
OF28-1	Meat PE	1/2a	*bcrABC*	Kovacevic et. al (2012a)
LR39-1*	Fish PE	1/2a	*emrE*	Kovacevic et. al (2012b)
LR59-1*	Fish PE	1/2b	*bcrABC*	Kovacevic et. al (2012b)
WRLP-354	Produce PE	Group 4	N/A	Jorgensen (2019)
WRLP-360	Produce PE	1/2a or 3a	N/A	Jorgensen (2019)
WRLP-367	Produce PE	1/2a or 3a	N/A	Jorgensen (2019)
WRLP-382*	Produce PE	Group 4	N/A	Jorgensen (2019)
L. seeligeri				
DE37-2	Dairy PE	N/A	N/A	Kovacevic et. al (2012a)
FE37-1	Fish PE	N/A	N/A	Kovacevic et. al (2012a)
L. welshimeri				
ME1-1	Meat PE	N/A	N/A	Kovacevic et. al (2012a)
FF6-3	Fish PE	N/A	N/A	Kovacevic et. al (2012a)

[1]S-AMR, antimicrobial resistance specific to sanitizer tolerance or resistance.
[2]Isolates were obtained from the culture collection of Dr. A. Yousef (Ohio State University), via Dr. Joy Waite-Cusic.
[3]Symbol '*' indicates isolates used in sub-lethal sanitizer growth experiments.

Table 3.2 Antimicrobials (n=4) tested in this study, including their active ingredients and concentrations, the manufacturer recommended concentrations and exposure time (MRC/ET), and the concentrations tested in minimum bactericidal concentration (MBC) assays.

Sanitizer	Active Ingredient(s)	Label MRC/ET[1]	Concentrations Tested in MBC Experiments (% of RTU[2] or MRC)
Alcohol-based (ALB) sanitizer	Alcohol	RTU /1 min[3]	100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78
Benzalkonium chloride (BAC)[4]	QUAT	200 ppm[5]/2 min	[6]50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39
Commercial QAC (CQAC)	QUAT	200 ppm/2 min	[6]50, 25, 12.5, 6.25, 3.13, 1.56, 0.78, 0.39
Citric acid-based (CAB) sanitizer	Citric acid Sodium dodecylbenzene sulfonate (SDS)	RTU/1 min[7]	100, 50, 25, 12.5, 6.25, 3.13, 1.56, 0.78

[1]MRC/ET, manufacturer recommended concentration and exposure time.
[2]RTU, ready-to-use solution.
[3]Active ingredient (ethyl alcohol) content in undiluted solution at 29.4% (v/v).
[4]Since benzalkonium chloride (BAC) is not a commercially used sanitizer, MRC/ET values were based on the recommendations for other QUAT sanitizers.
[5]Ppm, parts per million, equivalent to 200 μg/ml.
[6]Corresponds to tested concentrations of 100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 μg/ml.
[7]Active ingredients in undiluted solution: citric acid content at 0.660% (w/v%); SDS at 0.036% (w/v%).

Varying concentrations of sanitizers were added to 96-well microtiter plates through a series of two-fold dilutions in TSB. For ALB and CAB sanitizers, concentrations tested ranged from manufacturer recommended concentrations (MRC) to 1/128 of the MRC. The starting concentration for both BAC and CQAC was 100 μg/ml, with the lowest concentration tested 1/256 of MRC (Table 3.2). Diluted cultures were added (5 μl) to appropriate wells for a final inoculum of approximately 5 log CFU/ml. The 96-well microtiter plates were incubated at 4, 15, 23, or 30°C for 24 h, with shaking (150 rpm; New Brunswick Scientific, Innova 2000, Edison, NJ). Following incubation, each well was plated by streaking (10 μl) onto TSA + 0.6% yeast extract plates (TSAYE; Acumedia), and incubated at 37°C for 24 h. The MBC was determined as the lowest concentration of sanitizer at which no growth on TSAYE plates was observed. Experiments for each isolate were performed in duplicate and repeated at least three times.

3.2.4 Growth in presence of sub-lethal concentrations of sanitizers

A set of eight *L. monocytogenes* isolates, representing a range of more and less susceptible isolates to four sanitizers tested, based on MBC values, were selected to assess their adaptation and growth potential when exposed to sub-lethal concentrations of sanitizers (Table 3.1). Sub-lethal concentrations were based on the MBC data, with concentrations 2-fold lower than the MBC of the most susceptible isolate selected for each sanitizer. Growth of *L. monocytogenes* in the presence of four sanitizers (Table 3.2) was assessed in a SpectraMax (Molecular Devices, Sunnyvale, CA) plate reader at 30°C. Briefly, single colonies were inoculated into 5 ml of TSB and incubated at 30°C, with shaking (150 rpm; Thermo Scientific, MaxQ4000, Waltham, MA). Following 16 h incubation, cultures were diluted in TSB containing appropriate concentrations of sanitizers (approximately 5 log CFU/ml in 10 ml final volume). Positive controls, consisting of TSB with 100 µl of culture (approximately 5 log CFU/ml in 10 ml final volume), were also included. The 10 ml tubes with cultures and sanitizer treatments were incubated at 30°C for 24 h. Prior to incubation, duplicate aliquots (200 µl) were transferred from each tube into specified wells of a single sterile 96-well microtiter plate (VWR Tissue Culture Plates, VWR, Radnor, PA). Control wells, consisting of TSB and appropriate sanitizer dilution, were also included. Optical density (OD_{600}) readings were measured every 30 minutes. Additionally, at time points 0, 1, 3, 5.5, 8, 10 and 24 h, each treatment from the 10 ml tubes was enumerated using the track dilution method described by Jett et. al (1997). Samples were serially diluted (1:10) in 0.1% peptone water (PW; Acumedia), plated side by side on TSAYE plates, and incubated at 37°C for 24 h.

Blank controls containing TSB or TSB with appropriate concentrations of the tested sanitizer were included in each run and these OD_{600} values were subtracted from OD_{600} values for strains containing respective treatments. The OD_{600} readings were fitted to growth curves to obtain the lag phase duration (LPD; h), maximum growth rate (MGR; increase in OD_{600}/h)), and maximum optical density (MOD; OD_{600}), using DMFit 3.0 Excel add-in program (ComBase; Computational Microbiology Research Group, Institute of Food Research, Colney, Norwich, United Kingdom), based on the models of Baranyi and Roberts (1994). Plate counts were used to confirm the correspondence between OD_{600} values and viable cell counts at time points that represented early logarithmic, late logarithmic, and late stationary growth phases (0, 1, 3, 5.5, 8, 10 and 24 h) at 30°C. Cell counts were also fitted with DMFit model to obtain LPD, MGR ($\Delta \log_{10}$ CFU/ml) and maximum cell counts ($\log_{10}$ CFU/ml) for ALB, and for any isolate that did not have

reliable OD_{600} values when exposed to BAC and CQAC. Each isolate and control were run in duplicate and at least three experiments were completed for each treatment.

3.2.5 Statistical analysis

Statistical analysis was performed using R (R 3.5.2, R Foundation for Statistical Computing, Vienna, Austria) and Prism (Prism 8, GraphPad, San Diego, CA). One-way ANOVA with Tukey's multiple comparisons test at $P < 0.05$ was used to assess differences in MBCs of a sanitizer at different temperatures, and to assess differences among average MBCs of all sanitizers at different temperatures. One-way ANOVA with Tukey's multiple comparisons test was also used to determine if differences in average LPD, MGR, and MOD among *L. monocytogenes* isolates were significant at $P < 0.05$. Unpaired two-way t-tests with Welch's correction was used to compare average MBCs of isolates with sanitizer antimicrobial resistance (S-AMR) related genes to isolates with unknown S-AMR profiles. Standard error of the mean (SEM) and standard deviation (SD) was calculated in Excel (Excel 16.16, Microsoft Corp., Redmond, WA).

3.3 Results

3.3.1 MBCs of traditional and eco-friendly sanitizers against planktonic *Listeria* spp.

The MBCs of all sanitizers tested were below the MRCs. MBC values obtained in this study are listed in Appendix Tables A.1–A.4. Since each sanitizer had different active ingredients, MBC values of the traditional and eco-friendly sanitizers tested were not directly compared. Instead, sanitizers were compared based on the percentage of the MRC (% MRC) that represents the MBC.

With the highest MBC at 4.20% MRC, CQAC required consistently lower % MRC compared to the other sanitizers tested, at all temperatures (Fig. 3.1). At 30°C, the MBCs for CQAC ranged from 0.65 to 6.25 μg/ml and averaged at 5.12 μg/ml. This was significantly lower compared to BAC MBC values, which ranged from 10.42 to 20.80 μg/ml and averaged at 16.27 μg/ml at 30°C ($P < 0.01$; Table A.5).

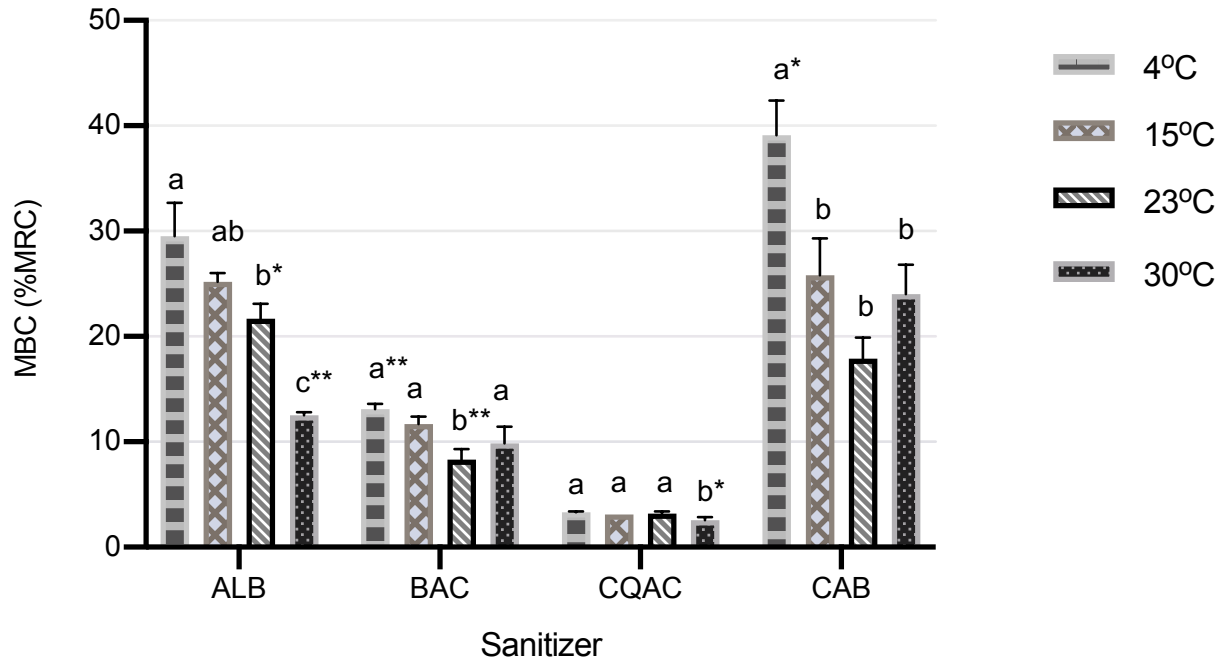

Figure 3.1 Average minimum bactericidal concentrations (MBC), reported as % of manufacturer recommended concentration (% MRC), of an alcohol-based (ALB) sanitizer, benzalkonium chloride (BAC), a commercial quaternary ammonium compound (CQAC), and a citric acid-based (CAB) sanitizer for *Listeria* spp. (n=22). Each sample was run in duplicate and experiments were repeated three times. Results represent the average MBC values of all isolates for each sanitizer from three independent experiments. Error bars represent standard error of the mean. Different letters next to bars represent significant differences between temperatures for each sanitizer but not across different sanitizer types assessed using one-way ANOVA with Tukey's multiple comparisons test at $P < 0.05$ (*) and $P < 0.01$ (**).

The average MBCs of both eco-friendly sanitizers required significantly higher % MRC when compared to average MBCs of CQAC and BAC at all temperatures tested ($P < 0.001$; Table A.6). Additionally, average MBCs of ALB were significantly higher than MBC values for CAB at 30ºC and 4ºC ($P < 0.001$). Average MBCs at 15ºC and 23ºC were also higher for ALB compared to CAB, but these values were not statistically significant ($P > 0.05$).

3.3.2 Effect of temperature on sanitizer MBCs against *Listeria* spp.

Temperature had a significant impact on the MBCs of all sanitizers tested ($P < 0.05$; Table 3.3). Overall, a decreasing trend was observed in MBCs as temperature increased (i.e. 23-30ºC). The highest average MBC for all sanitizers was obtained at 4ºC. However, the lowest average MBC did not consistently occur at 30ºC. The lowest average MBC for ALB and CQAC was

observed at 30°C, whereas the lowest values for BAC and CAB were seen at 23°C. Analysis of individual isolate MBC values revealed that the lethality of CQAC was the least affected by temperature (Table A.3).

Table 3.3. Average minimum bactericidal concentrations (MBCs) of an alcohol-based (ALB) sanitizer, benzalkonium chloride (BAC), a commercially available quaternary ammonium compound (CQAC), and a citric acid-based (CAB) sanitizer at temperatures ranging from 4 to 30°C, reported as % of manufacturer recommended concentrations (% MRC) against *Listeria* spp. (n=22). Experiments were performed in duplicate for each isolate and repeated three times. Results represent the average MBCs and standard error of the mean (SEM) of all isolates from three independent experiments.

	Average Minimum Bactericidal Concentration (%MRC ± SEM)[1]			
Sanitizer	**4°C**	**15°C**	**23°C**	**30°C**
ALB	29.50 ± 3.20a	25.20 ± 0.80ab	21.70 ± 1.4b*	12.50 ± 0.30c**
BAC	13.10 ± 0.50a**	11.70 ± 0.70ab	8.30 ± 1.00b**	9.85 ± 1.60ab
CQAC	3.30 ± 0.10a	3.10 ± 0.00a	3.20 ± 0.20a	2.56 ± 0.29b*
CAB	39.10 ± 3.30a*[2]	25.80 ± 3.50b	17.90 ± 2.00b	24.00 ± 2.80b

[1]Values from sanitizers were analyzed using ANOVA and Tukey's multiple comparisons test. Different letters indicate significant differences at $P < 0.05$ (*) and $P < 0.01$ (**) in MBC values for each sanitizer at different temperature; however, values were not compared between sanitizers.
[2]P values of CAB MBCs at 4°C were significantly different from those at 23°C and 30°C at $P < 0.001$ and $P < 0.01$, respectively.

3.3.3 Difference in MBCs amongst *Listeria* spp. isolates

Differences in MBC values between isolates were observed, with the most variability seen with CAB treatment (Table A.4). Isolate *Lm*-Ohio was particularly susceptible to CAB. It had MBC values ranging from 8 times lower at 4°C to 20 times lower at 30°C when compared to the average MBC value of all 22 *Listeria* spp. at both respective temperatures (Fig. 3.2).

Six of the isolates possessed S-AMR related genes (Table 3.1). With the exception of one isolate with the S-AMR gene deletion (08-5578_Δ1862), presence of S-AMR genes in the remaining 15 isolates was unknown.

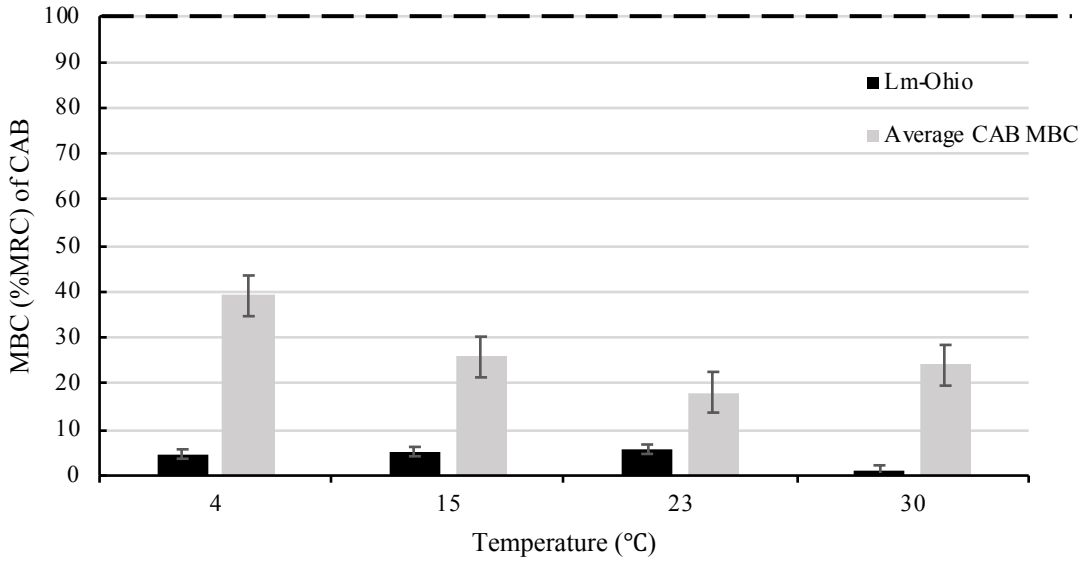

Figure 3.2. Minimum bactericidal concentrations (MBCs; reported as % manufacturer recommended concentration [MRC]) of a citric acid-based (CAB) sanitizer for *L. monocytogenes* isolate Ohio compared to the overall average MBC of CAB for all *Listeria* spp. (n=22) tested. The MRC is indicated by the dashed line. Experiments were completed three times, with each isolate run in duplicate. Results represent the average MBCs of all isolates from three independent experiments. Error bars represent standard error of the mean.

Overall, the average MBC of BAC at all temperatures was higher for isolates possessing S-AMR genes compared to the average MBC of 08-5578_Δ1862 and isolates with unknown AMR gene profiles, but these differences were not statistically significant ($P > 0.05$; Table A.7). Average MBCs of ALB and CAB for S-AMR possessing isolates were significantly lower ($P < 0.001$) and significantly higher at 4°C ($P < 0.05$), respectively, compared to isolates with unknown AMR (Fig. 3.3). Despite these differences, no clear trends of increased tolerance in either group was observed based on the MBC data (Table A.7). This may be in part due to the presence of S-AMR genes in the "unknown" group of isolates or due to the experiments not being sensitive enough to detect differences in MBC values with the series of 2-fold diluted sanitizer concentrations tested. This is supported by MBC results of isolate 08-5578_Δ1862, a mutant of 08-5578, with *emrE* gene deleted. Although differences in the susceptibilities of these isolates were expected to be apparent for QUATs, no significant differences in MBCs of BAC or CQAC were seen ($P > 0.05$; Table A.8).

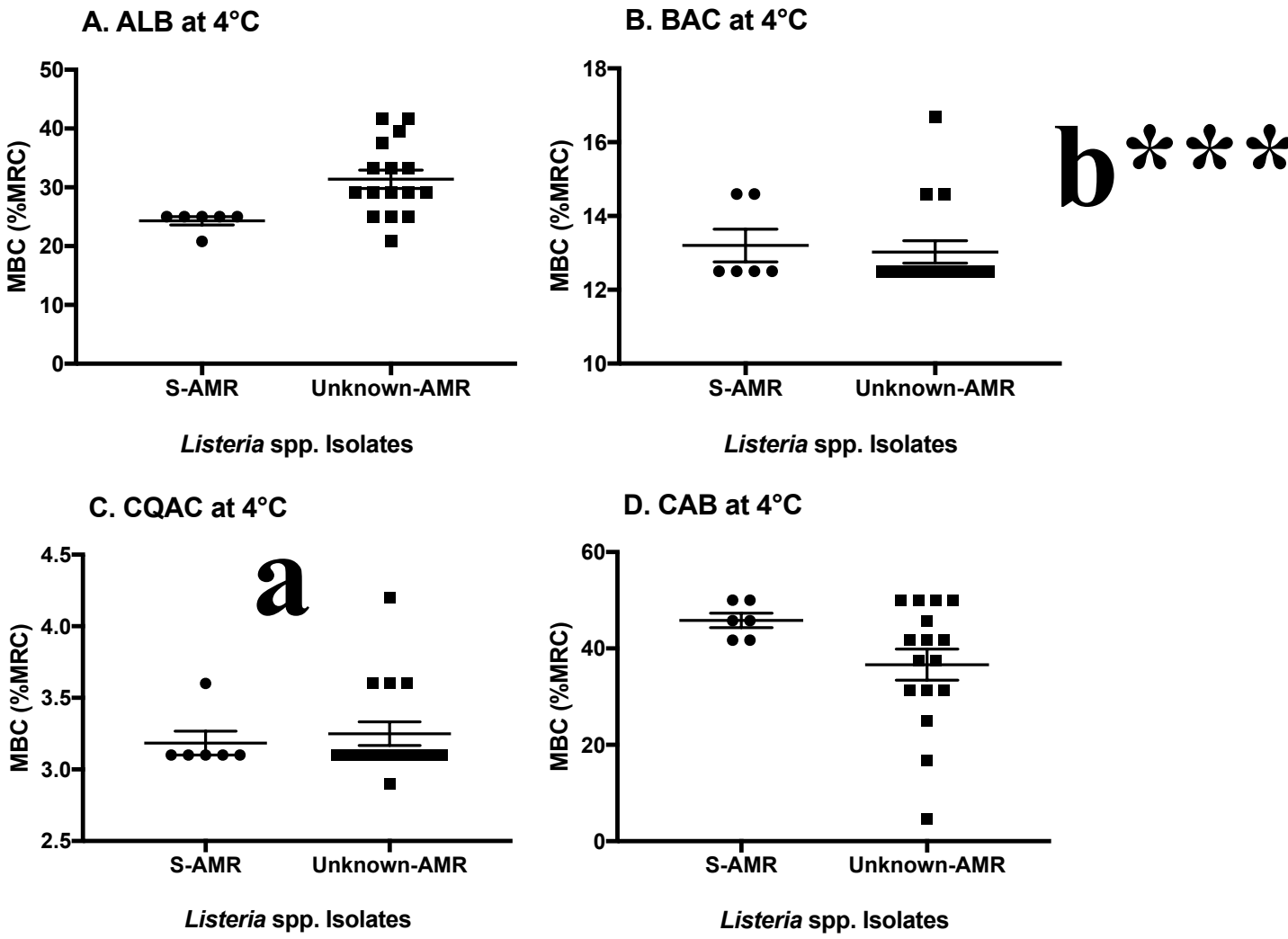

Figure 3.3. Minimum bactericidal concentrations (MBCs) of (A) an alcohol-based (ALB) sanitizer, (B) benzalkonium chloride (BAC), (C) a commercial quaternary ammonium compound (CQAC), and (D) a citric acid-based (CAB) sanitizer at 4ºC presented as % manufacturer recommended concentration (%MRC) against *Listeria* spp. isolates with sanitizer antimicrobial resistance genes (S-AMR; n=6) and isolates where S-AMR is unknown (n=16). Experiments were performed in duplicate for each isolate and repeated three times. Results were analyzed using unpaired t-test with Welch's correction, where different letters indicate significant differences at $P < 0.05$ (*) and $P < 0.001$ (***).

3.3.4 Effect of sub-lethal sanitizers concentrations on growth curves of *L. monocytogenes* isolates

The growth of eight *L. monocytogenes* isolates in the presence of sub-lethal sanitizer concentrations was assessed over 24 h using both OD_{600} and enumeration methods (log CFU/ml).

The OD_{600} data was fitted to obtain LPD, MGR and MOD for all treatments except for isolates grown in ALB (Table 3.4). Upon mixing ALB with TSB, precipitate formed, clouding the solution and rendering OD_{600} readings unreliable. Growth of isolates exposed to sub-lethal concentrations of ALB sanitizer were evaluated using plate counts only (Table 3.5).

When exposed to sub-lethal sanitizer stress, average LPDs for all isolates and all treatments were longer than those of the positive control (i.e. isolates grown in TSB only), with LPDs for isolates grown in CAB lasting up to 6 h longer. Based on OD_{600} values, there were no significant differences in LPDs among six isolates for any sanitizer treatment ($P > 0.05$; Table 3.4). However, isolates 08-5578_Δ1862 and WRLP-382 were severely impaired at tested sub-lethal concentrations of BAC and CQAC, with no OD_{600} values collected. These isolates appeared to be similarly affected by BAC and CQAC, with no growth observed when both isolates were exposed to CQAC, and severely impaired growth seen for 08-5578_Δ1862 when exposed to BAC (Fig. 3.4). The log CFU/ml data showed that growth did not start for these two isolates within 24 h (Table A.10 – A.11), indicating these concentrations were too high.

There were no significant differences between MGRs of isolates when exposed to BAC and CAB ($P < 0.05$). Similar to isolates 08-5578_Δ1862 and WRLP-382, isolate LR39-1 and LR-59-1 had a significantly lower MGR in CQAC compared to the MGRs of other isolates tested ($P < 0.01$). However, LR39-1 did not have a significantly different MOD, suggesting this isolate reached similar cell density as the other isolates tested. Enumeration data confirmed that cell counts for all isolates, except for those severely affected by CQAC (i.e. 08-5578_Δ1862 and WRLP-382), were not significantly different at 24 h ($P < 0.05$; Table A.11). Isolate LR59-1 had a significantly lower average MOD value in the presence of sub-lethal CQAC ($P < 0.05$), which was not reflected in CFU counts. Growth curves indicated that LR39-1 and LR59-1 followed similar growth patterns as other isolates, excluding 08-5578_Δ1862 (Fig. 3.5).

Table 3.4. Average lag phase duration (h), maximum growth rate (increase in OD_{600}/h), and maximum optical density (OD_{600}) of *L. monocytogenes* (*Lm*) isolates (n=8), when exposed to sub-lethal concentrations of benzalkonium chloride (BAC), a commercial quaternary ammonium compound (CQAC), and citric acid-based (CAB) sanitizer for 24 h at 30°C. Values represent mean values (± standard deviation) from three independent assays, with each sample and treatment measured in duplicate.

***Lm* Isolate**	**BAC[1] (3.13% MRC[2])**	**CQAC (0.78% MRC[3])**	**CAB (6.25% MRC)**	**Positive Control**
Lag phase duration (h)				
OSY-428	10.53 ± 1.77a	8.65 ± 0.84a	14.36 ± 3.06a	7.95 ± 0.67a
08-5578	13.63 ± 6.19a	8.86 ± 0.29a	13.99 ± 3.48a	7.94 ± 0.49a
08-5578_Δ1862	N/A[4]	N/A	13.85 ± 3.50a	7.85 ± 0.57a
FE7-1	10.51 ± 2.15a	8.56 ± 0.46a	11.16 ± 2.03a	8.04 ± 0.60a
FE10-1	10.64 ± 2.28a	8.70 ± 0.47a	11.12 ± 2.17a	7.86 ± 0.82a
LR39-1	13.71 ± 3.90a	9.25 ± 0.35a	12.69 ± 1.66a	7.97 ± 0.62a
LR59-1	12.00 ± 4.96a	8.39 ± 0.92a	11.66 ± 2.79a	7.76 ± 0.83a
WRLP-382	N/A[4]	N/A	10.89 ± 2.00a	7.82 ± 0.73a
Maximum growth rate (increase in OD_{600}/ h)				
OSY-428	0.13 ± 0.07a	0.18 ± 0.01a	0.10 ± 0.13a	0.22 ± 0.02a
08-5578	0.05 ± 0.07a	0.16 ± 0.01a	0.12 ± 0.14a	0.22 ± 0.00a
08-5578_Δ1862	0.04 ± 0.06a	N/A	0.03 ± 0.01a	0.21 ± 0.00ac*
FE7-1	0.18 ± 0.04a	0.17 ± 0.01a	0.04 ± 0.01a	0.21 ± 0.01a
FE10-1	0.12 ± 0.05a	0.15 ± 0.01ab**	0.05 ± 0.01a	0.16 ± 0.01b*
LR39-1	0.13 ± 0.06a	0.13 ± 0.01bc*	0.03 ± 0.01a	0.18 ± 0.00c*
LR59-1	0.16 ± 0.06a	0.12 ± 0.02c**	0.03 ± 0.01a	0.19 ± 0.01c*
WRLP-382	N/A	N/A	0.06 ± 0.01a	0.17 ± 0.01bc*
Maximum optical density (OD_{600})				
OSY-428	0.61 ± 0.01a	0.64 ± 0.01ac*	0.24 ± 0.04a*	0.69 ± 0.01a
08-5578	0.43 ± 0.30ab	0.65 ± 0.04ac***	0.28 ± 0.07ab	0.68 ± 0.01a
08-5578_Δ1862	0.10 ± 0.18bc*	0.002 ± 0.00b***	0.28 ± 0.11ab	0.69 ± 0.01a
FE7-1	0.63 ± .031a	0.67 ± 0.01a	0.33 ± 0.02ab	0.68 ± 0.02a
FE10-1	0.63 ± 0.03a	0.64 ± 0.00ac*	0.42 ± 0.02b*	0.64 ± 0.03a
LR39-1	0.51 ± 0.10a	0.56 ± 0.03cd*	0.29 ± 0.04ab	0.67 ± 0.02a
LR59-1	0.58 ± 0.06a	0.53 ± 0.08d*	0.33 ± 0.04ab	0.67 ± 0.02a
WRLP-382	N/A	N/A	0.37 ± 0.04ab	0.66 ± 0.01a

[1]Statistically significant values within each treatment are indicated with different letters (vertical comparison only) at $P < 0.05$ (*), $P < 0.01$ (**), $P < 0.001$ (***), using one-way ANOVA with Tukey's multiple comparisons test.

[2]Sub-lethal concentration used for BAC was 6.25 μg/ml

[3]Sub-lethal concentration used for CQAC was 1.56 μg/ml

[4]N/A, not available as OD_{600} values were unreliable due to lack of growth; confirmed through log CFU/ml counts.

Table 3.5. Average lag phase duration, maximum growth rate, and maximum optical density of *L. monocytogenes* (*Lm*) isolates (n=8), when exposed to sub-lethal concentrations of an alcohol-based sanitizer (ALB; 6.25 manufacturer recommended concentration [MRC]) at 30°C. Values represent mean values (± standard deviation) from three independent assays, with each sample and treatment measured in duplicate.

***Lm* Isolate**	**LPD (h)[1,2]**	**MGR (Δlog_{10} CFU/ml)**	**MOD (log_{10} CFU/ml)**
OSY-428	10	0.029 ± 0.008a**	0.636 ± 0.008a*
08-5578	10	0.012 ± 0.005ac	0.592 ± 0.007ab
08-5578_Δ1862	10	0.011 ± 0.008ac	0.605 ± 0.011ab
FE7-1	No growth[3]	-0.013 ± 0.014b**	0.596 ± 0.049ab
FE10-1	No growth	-0.010 ± 0.007bc*	0.554 ± 0.029b*
LR39-1	10	0.010 ± 0.006ac	0.593 ± 0.034ab
LR59-1	10	0.015 ± 0.009ac	0.600 ± 0.023ab
WRLP-382	10	0.016 ± 0.011a*	0.625 ± 0.014ab

[1]Statistically significant values within each treatment are indicated with different letters (vertical comparison only) at $P < 0.05$ (*) and $P < 0.01$ (**) using one-way ANOVA with Tukey's multiple comparisons test.
[2]Values were calculated from CFU/ml counts at 0, 1, 3, 5.5, 8, 10, and 24 h.
[3]LPD values unavailable due to lack of growth indicated by CFU/ml counts.

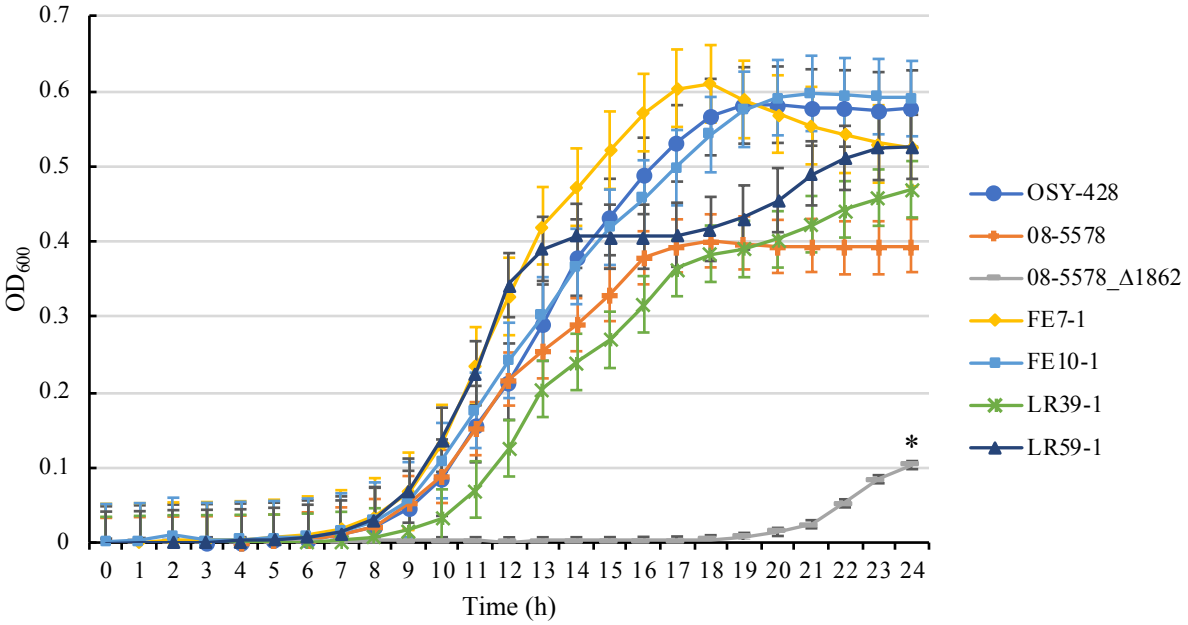

Figure 3.4. Growth of seven *L. monocytogenes* isolates in tryptic soy broth with sub-lethal concentrations of benzalkonium chloride (BAC; 6.25 µg/ml) at 30°C for 24 h. Each isolate was run in duplicate and results represent average optical density (OD_{600}) from three independent experiments. Error bars represent standard error of the mean. Isolates 08-5578_Δ1862 and WRLP-382 showed impaired and no growth, respectively, indicated with (*).

Based on OD_{600} data for BAC, CQAC and CAB, isolates responded the most uniformly when treated with CAB (Fig. 3.6). In the presence of sub-lethal CAB concentration, the highest

MOD values were seen for isolates WRLP-382 and FE10-1, with FE10-1 significantly higher compared to other isolates ($P < 0.05$; Table 3.4). Data from log CFU/ml counts showed that, while WRLP-382 had the highest growth, FE10-1 had significantly lower counts compared to WRLP-382 ($P < 0.05$; Table A.12). WRLP-382 also had significantly higher counts compared to 08-5578_Δ1862 ($P < 0.05$), but it was not statistically different from other isolates.

When exposed to sub-lethal concentrations of ALB, isolates exhibited similar growth patterns, with the exception of isolates FE7-1 and FE10-1 (Fig. 3.7). These two isolates did not grow within the 24 h. Instead, MGRs (Δlog CFU/ml/h) indicated that populations of these two isolates were slowly declining (Table 3.5). At 24 h, log CFU/ml counts showed that these two isolates were not significantly different from each other, but they were significantly different from every other isolate tested, indicating slightly increased sensitivity to ALB ($P < 0.05$; Table A.9).

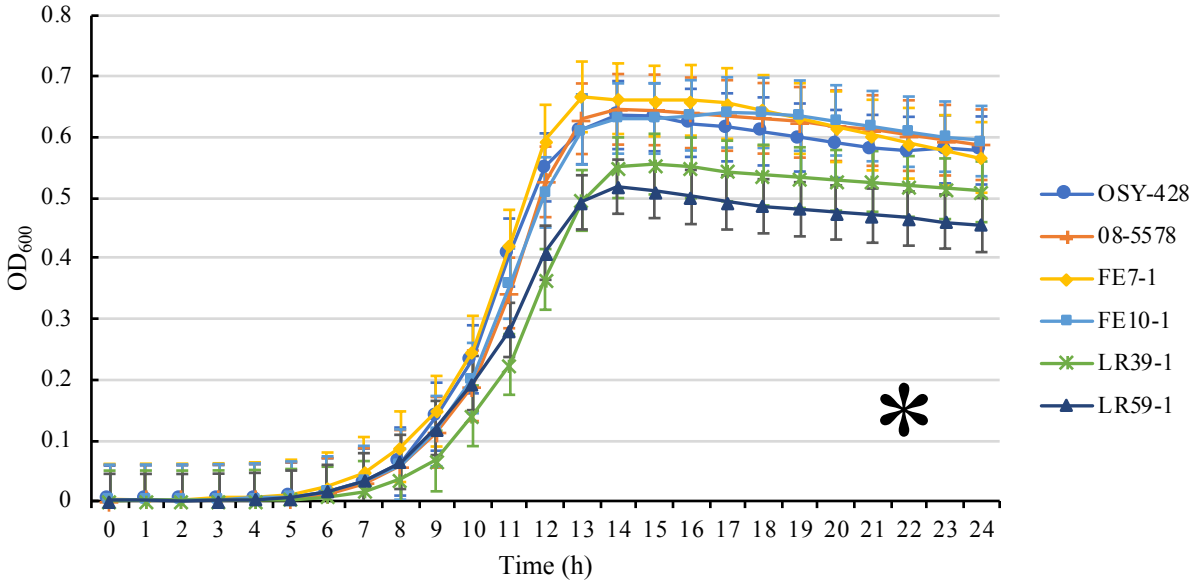

Figure 3.5. Growth of six *L. monocytogenes* isolates in tryptic soy broth with sub-lethal concentrations of a commercial quaternary ammonium compound (CQAC; 1.56 µg/ml) at 30ºC for 24 h. Each isolate was run in duplicate and results represent average optical density (OD_{600}) from three independent experiments. Error bars represent standard error of the mean. *Isolates 08-5578_Δ1862 and WRLP-382 were excluded from the graph as they exhibited no growth at the concentration tested.

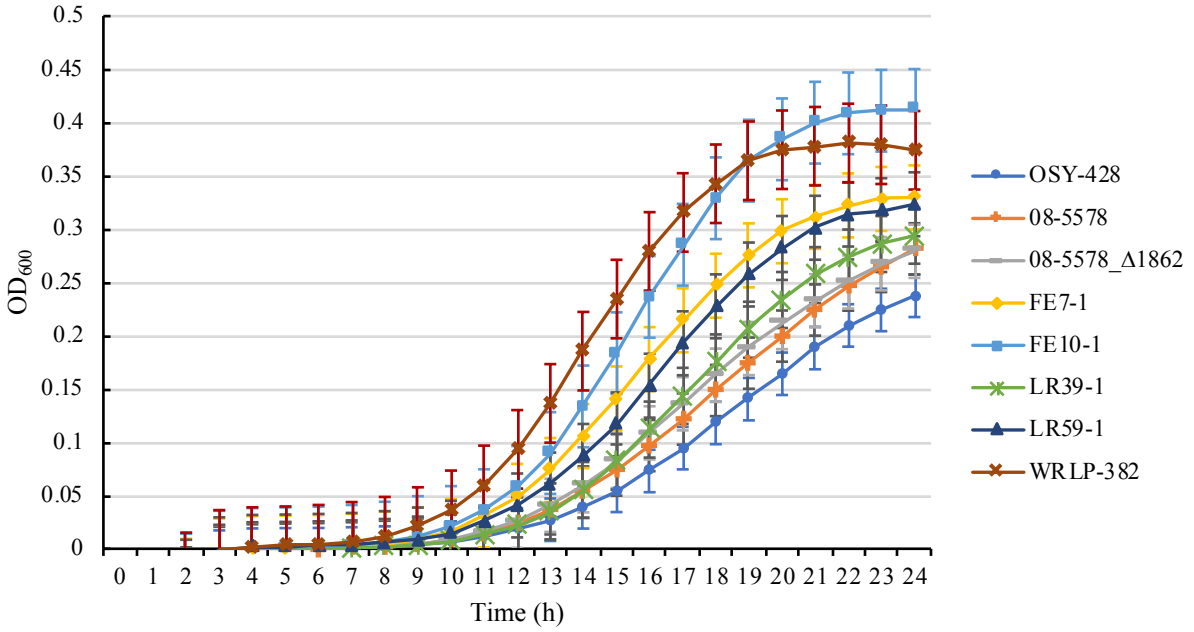

Figure. 3.6. Growth of eight *L. monocytogenes* isolates in tryptic soy broth with sub-lethal concentrations of a citric acid-based (CAB; 6.25% manufacturer recommended concentration [MRC]) sanitizer at 30°C for 24 h. Each isolate was run in duplicate and results represent average optical density (OD_{600}) from three independent experiments.

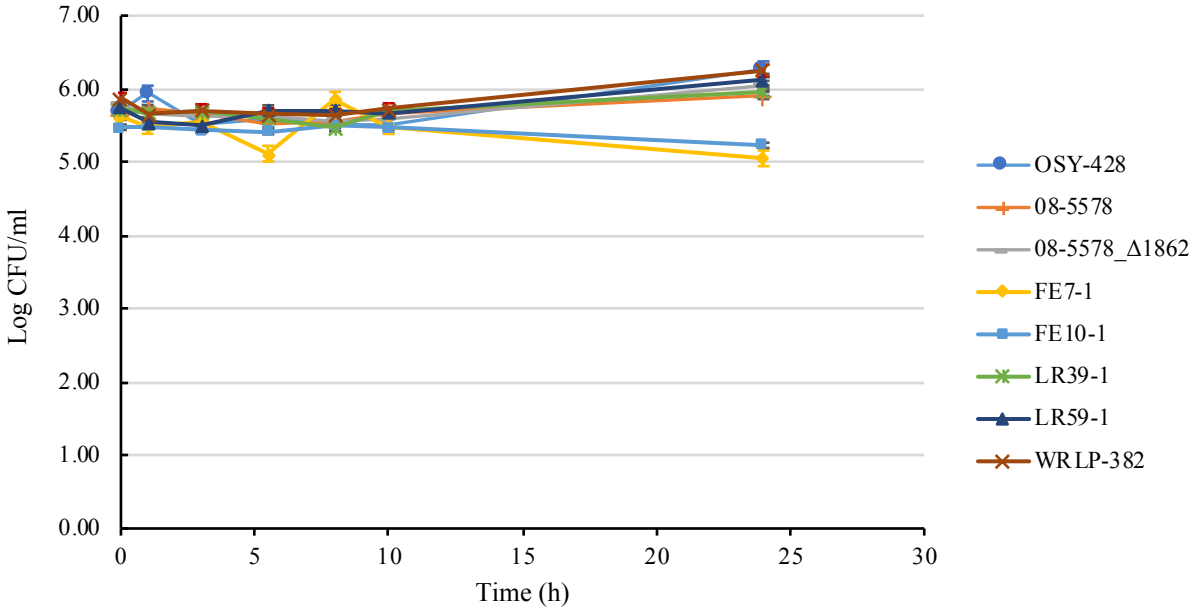

Figure 3.7. Growth of eight *L. monocytogenes* isolates in tryptic soy broth with sub-lethal concentrations of an alcohol-based (ALB; 6.25% manufacturer recommended concentration) sanitizer at 30°C for 24 h. Each isolate was run in duplicate and results represent average CFU/ml counts from three independent experiments. Error bars represent standard error of the mean.

3.4 Discussion

The MBCs of ALB, BAC, CQAC, and CAB against 22 *Listeria* spp. isolates at temperatures ranging from 4 to 30°C revealed that all sanitizers tested were capable of controlling planktonic *Listeria* spp. at the MRC. Comparing % MRC, sanitizers with eco-friendly ingredients (ALB and CAB) had overall significantly higher average MBCs than the traditional sanitizers ($P < 0.001$), with the exception of exposures to ALB and BAC at 30°C ($P > 0.05$; Table A.6). The MBCs of the CQAC sanitizer were significantly lower than all other sanitizers at each temperature tested ($P < 0.05$; Table A.6). While sanitizers must be used according to the label, our MBC data indicate that the CQAC sanitizer can withstand more user error while still being effective against *Listeria* spp. compared to the other sanitizers tested.

When sanitizers were applied at different temperatures, increased MBC values were observed as temperature decreased (Fig. 3.1). Similar results were reported by Tuncan (1993). Specifically, decreased germicidal activity of a QUAT, iodophor, and chlorine sanitizer against planktonic *Listeria* spp. with a 30 sec exposure time was observed at 2°C compared to 25°C (Tuncan, 1993). Although it was the least affected by temperature, the MBCs of the CQAC sanitizer tested here were significantly lower at 30°C compared to 4°C ($P < 0.05$; Table 3.3). While this observation may be in part due to the sanitizer itself being affected, studies have also reported physiological changes in cold-adapted *L. monocytogenes* that may lead to increased tolerance to other stresses, including antibiotic, alkali and oxidative stress (Abeysundara et al., 2019; Al-Nabulsi et al., 2015). As exposure to low temperatures activates sigma-B (σ^B), the main component and regulator in *L. monocytogenes* stress response network, protection against a wide range of stress conditions, including antimicrobials is not surprising (Becker et al., 2000; van Schaik and Tjakko, 2005).

While the current study did not utilize molecular approaches to elucidate potential genetic differences between isolates that may explain variation in MBCs and growth behavior in the presence of sub-lethal concentrations of sanitizers, other studies have described roles of σ^B regulated genes (Tamburro et al., 2015) and other genetic determinants that confer resistance to QUAT sanitizers, such as efflux pumps encoded by *emrE* (Kovacevic et al., 2016) and *bcrABC* (Elhanafi et al., 2010). Additionally, some multidrug efflux pumps have been found to provide a nonspecific increase in tolerance to bactericides (Alcalde-Rico et al., 2016; Romanova et al., 2006; Sidhu et al., 2001). Our data showed this was true for six S-AMR *L. monocytogenes* isolates when

exposed to CAB at 4°C. Isolates that possessed known efflux pumps exhibited higher MBCs compared to MBCs of 16 isolates with no/unknown S-AMR profiles ($P < 0.05$) (Fig. 3.3). In contrast, isolates with efflux pumps showed increased sensitivity to ALB ($P < 0.001$), for reasons not apparent.

Notably, *L. monocytogenes* isolate Ohio, belonging to serotype 4b, was particularly susceptible to CAB. Although *L. monocytogenes* as a species is considered to be relatively acid tolerant, discrepancies in tolerance across strains has been observed (Francis and O'Beirne, 2005; Hingston et al., 2017; Phan-Thanh et al., 2000). Different genetic factors, specifically differences in a number of putative σ^B-regulated genes, have been proposed to play a role in acid tolerance in *L. monocytogenes* (Kazmierczak et al., 2003; Sue et al., 2004; Wiedmann et al., 1998). Ryan et al. (2010) also discovered a collection of five genes that aid in the survival of *L. monocytogenes* in acidic conditions, collectively referred to as stress survival islet-1 (SSI-1). Notably, their analysis of 45 *L. monocytogenes* isolates revealed that this islet was present in 51% of the isolates, with no SSI-1 seen in serogroup 4 (Ryan et al., 2010). As isolate Ohio belongs to serogroup 4, future genomic studies will explore genetic elements involved in acid stress response in this strain.

Listeria monocytogenes isolates with S-AMR did not have significantly higher BAC and CQAC MBCs compared to unknown S-AMR group, at all temperatures tested (Table A.7). This was further confirmed with MBC values of isolates 08-5578 and its Δ*emrE* mutant (08-5578_Δ1862), when exposed to two QUATs. It was expected that isolate 08-5578 would have higher MBCs compared to its Δ*emrE* mutant; however, no significant differences were seen ($P > 0.05$; Table A.8). This may be due to insufficient sensitivity of MBC assay used, with 2-fold dilutions between each concentration tested not being sensitive enough to show minor discrepancies in sanitizer tolerance of isolates, or due to the isolate differences being negligible. Additionally, since no S-AMR information was available for 15 isolates tested here it is possible that some of the isolates have known or novel S-AMR properties, resulting in inflated average MBCs (Fig. 3.3). Future studies will focus on comparison of a larger set of isolates with the confirmed presence and absence of known S-AMR.

Similar to MBC data, when a subset of *L. monocytogenes* isolates (n = 8) were exposed to sub-lethal concentrations of BAC, CQAC, ALB, and CAB, variation in growth behavior among isolates was observed with all sanitizers tested. Maximum growth rates, based on the log CFU/ml counts, of two isolates (FE7-1 and FE10-1) showed that they were significantly more affected by

ALB compared to other isolates tested ($P < 0.05$; Table 3.5). Overall, *L. monocytogenes* response to ethanol, the active ingredient in ALB, is not fully understood. Exposure to ethanol activates σ^B, and the σ^B regulated gene *hfq* is believed to aid in protecting the cell against ethanol, however, Ferreira et al. (2001) found that exposing σ^B deletion mutant to 16.5% ethanol did not show significant difference in viability compared to the wild-type strain, indicating that other mechanisms also contribute to ethanol stress resistance (Christiansen et al., 2004; Ferreira et al., 2001). Future studies will explore genomic differences in FE7-1 and FE10-1 strains that may be associated with ethanol susceptibility.

When exposed to sub-lethal concentrations of BAC and CQAC, isolates that possessed *emrE* (n=2) and *bcrABC* (n=3) behaved similarly, with no trend of increased tolerance seen in isolates with *emrE* compared to *bcrABC*, and vice versa (Figs. 3.4-3.5). In addition, isolate OSY-428 exhibited growth characteristics similar to isolates with confirmed efflux pumps genes when grown in the presence of sub-lethal BAC and CQAC (Figs. 3.4-3.5). As expected, isolate 08-5579_Δ1862, *emrE* deletion mutant, was more prominently affected by BAC and CQAC (Kovacevic et al., 2016). Similarly, WRLP-382, an isolate that has been recovered from a produce processing facility in the U.S., was also inhibited by sub-lethal concentrations of two tested QUATs. Studies are currently underway to elucidate mechanisms behind decreased and increased sensitivities of OSY-428 and WRLP-382 isolates, respectively, to QUATs.

In summary, while isolates tested here were all susceptible to BAC, CQAC, ALB, and BAC applied at MRCs, variability in MBCs and growth behavior in the presence of sub-lethal stresses was observed. Traditional sanitizers effectively inactivated various *Listeria* spp. at lower percentages of the MRC compared to eco-friendly sanitizers. Notably, all sanitizers were significantly affected by changes in temperature, with decreased efficacy observed at higher (23 and 30ºC) compared to lower temperatures (4ºC). The lethality of the CQAC sanitizer was the least affected by temperature. Given that differences in growth in the presence of sub-lethal concentrations of sanitizers were observed among different isolates, adequate application of sanitizers in food processing facilities is critical to avoid treatment of areas with sub-lethal sanitizer stress. Sub-lethal sanitizer levels in the processing environment, due to incorrect preparation, unintentional dilution within the processing area, or inadequate application on surfaces, have been shown to promote stress adaptation in *L. monocytogenes* and lead to reduced susceptibilities not only to the sanitizer itself but also to antibiotics and other antimicrobials (Kovacevic et al., 2013;

Lunden et al., 2003). Collectively, this study emphasizes the need for rigorous cleaning and sanitation regimes in food processing facilities that take into account processing environment conditions, such as sanitizer application temperatures, specific to the facility.

3.5 Acknowledgments

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Chapter 4: Determining the Efficacy of Traditional and Eco-Friendly Sanitizers at Controlling *L. monocytogenes* When Attached to Common Food Contact Surface Materials

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Abstract:

The ability of *Listeria monocytogenes'* (*Lm*) to attach and form biofilms on surfaces found in food processing facilities poses a challenge for effective cleaning and sanitation. Eco-friendly (EF; n=2) and traditional (TR; n=2) sanitizers were tested against *Lm* isolates (n=5) attached to stainless steel (SS) and plastic coupons (1.3cmx1.3cm) at 4°C or 30°C (24h), when sanitizers were applied according to manufacturer recommendations and in the presence or absence of cleaning prior to sanitizing. Without cleaning, at least 2/10 replicates survived following attachment at 30°C, and 56.3% were positive ≥5/10 times in all sanitizer and temperature treatments. Increased inactivation was observed when attachment occurred at 4°C (124/400) compared to 30°C (235/400), and when *Lm* were attached to plastic. Scanning electron micrographs of WRLP-360 showed lower *Lm* counts on coupons attached at 4°C compared to 30°C. Plastic coupons appeared smoother than SS, which likely accounted for the decreased survival observed on plastic. The addition of a cleaning step did not decrease the incidence of survival of *Lm* attached to SS coupons at 30°C; however, it led to a decrease in *Lm* survival on plastic coupons and specifically, coupons treated with EF sanitizers. This work highlights the importance of frequent cleaning and sanitation.

Keywords: *Listeria monocytogenes*, eco-friendly sanitizers, attachment, stainless steel, plastic, cleaning

Highlights:

- Improved sanitizer inactivation of *L. monocytogenes* cells attached at 4°C vs. 30°C
- Reduced survival of *Lm* on plastic vs. stainless steel after sanitizer treatment
- Cleaning step improved inactivation on plastic for eco-friendly sanitizers only

4.1 Introduction

Listeria monocytogenes is a foodborne pathogen responsible for causing severe illness in at-risk populations. With 20–30% mortality rates associated with infections in susceptible individuals, primarily elderly, neonates, pregnant women, and immunocompromised persons, it is imperative to keep *L. monocytogenes* from contaminating ready-to-eat (RTE) foods (Camejo et al., 2011).

The challenge with controlling *L. monocytogenes* in food processing environments and preventing food contamination is linked to the ability of these microorganisms to form biofilms, and tolerate high salinity, low pH, and low temperature conditions often encountered in food processing facilities (Swaminathan and Gerner-Smidt, 2007; Vogel et al., 2010). When conditions are favorable, these organisms have been found to persist in food processing environments for years and even decades (Ferreira et al., 2014). Orsi et al. (2008) described a strain of *L. monocytogenes* that had persisted in a meat processing facility for at least 12 years. In 1988, this strain caused a sporadic listeriosis case, linked to contaminated turkey franks. Twelve years later, it was responsible for an outbreak associated with delicatessen meat. The ability of *L. monocytogenes* to survive and persist in processing environments allows for these areas to act as a reservoir of *L. monocytogenes* (Farber and Peterkin, 1991). In a study that looked at the persistence of *L. monocytogenes* in fish slaughter and smokehouse operations, Wulff et al. (2006) found *L. monocytogenes* strains that persisted for the entire one and half years of their project. They also found that, while none of the raw fish they sampled had *L. monocytogenes*, 27% of the processed samples tested positive, confirming the contamination was acquired during or post-processing. Samples taken from food contact and non-food contact surfaces also tested positive for *L. monocytogenes* even immediately after the surfaces were cleaned and sanitized (Wulff et al., 2006).

While cleaning and sanitation is one of the key measures in controlling environmental pathogens and preventing contamination of foods from processing environment, cleaning and sanitizing regimes are not always effective at completely eliminating *L. monocytogenes* (Krysinski et al., 1992; Wulff et al., 2006). The process of sanitizing processing areas requires the surface to be rinsed, cleaned by scrubbing the surface with a detergent, rinsed again, and then sanitized following recommendations found on the sanitizer label. Hard to clean areas that are not regularly scrubbed or adequately treated with sanitizer may permit bacterial attachment and colonization

(Krysinski et al., 1992; Wulff et al., 2006). It is well documented that attached bacteria, and especially those in biofilms, are more tolerant to a variety of stressors, including sanitizers (Chavant et al., 2004; Davies, 2003; Frank and Koffi, 1990).

The ability of *L. monocytogenes* to attach to surfaces is believed to be affected by temperature, with lower levels of attachment occurring as temperature decreases (Herald and Zottola, 1988; Mai and Conner, 2007; Smoot and Pierson, 1998). Additionally, the surface material can affect the rate of bacterial attachment (Silva et al., 2008; Smoot and Pierson, 1998). Several studies have tested the ability of sanitizers against *L. monocytogenes* attached to commonly used food contact surface materials, including stainless steel (SS), nitrile rubber, and plastic conveyor belts (Krysinski et al., 1992; Smoot and Pierson, 1998). Attachment to these materials differed both in the abundance of *L. monocytogenes* and the attachment strength. Surface topography and physiochemical interactions between the bacteria and surface material are thought to play a role in attachment, though precise mechanism are unclear (Mafu et al., 1990; Silva et al., 2008).

With the widespread use of QUATs and increased availability of sanitizers with eco-friendly ingredients, the goal of this study was to test if sanitizers are capable of inactivating *L. monocytogenes* that have been attached to common food contact surface materials when sanitizers were applied following the manufacturer recommended concentrations and exposure times (MRC/ET), at two different temperatures (4°C and 30°C). An additional objective was to explore whether a cleaning step prior to sanitizing effectively reduced incidence of survival of attached *Lm* on both SS and plastic surfaces.

4.2 Materials and methods

4.2.1 Bacterial isolates

A selection of five *L. monocytogenes* isolates with the highest tolerance to all antimicrobials tested as determined by Boucher et al. (2019), were used in this study (Table 4.1). Isolates were stored in a trypticase soy broth (TSB; Acumedia, Lansing, MI) and 50% glycerol solution at -80^{o}C. Prior to use, isolates were activated by plating frozen stocks onto trypticase soy agar (TSA; Acumedia) supplemented with 5% defibrinated sheep blood (BAP; Hardy Diagnostics, Santa Maria, CA) and incubated at 37^{o}C for 24 h. BAP plates were stored at 4^{o}C and used for a maximum of two weeks.

Table 4.1. Minimum bactericidal concentrations (MBC) of an alcohol-based (ALB) sanitizer, benzalkonium chloride (BAC), a commercially available quaternary ammonium compound (CQAC), and a citric acid-based (CAB) sanitizer at 30°C against *L. monocytogenes* (*Lm*) isolates (n=5). Results are presented as % of the manufacturer recommended concentration (%MRC).

	MBC (% MRC) at 30°C			
***Lm* Isolate**	**BAC**	**CQAC**	**ALB**	**CAB**
OSY-428	12.5%	3.1%	12.5%	12.5%
08-5578	9.4%	2.6%	12.5%	25.0%
LR59-1	12.5%	2.6%	12.5%	43.8%
WRLP-354	6.3%	2.6%	12.5%	33.3%
WRLP-360	7.3%	2.9%	12.5%	16.7%

4.2.2 Preparation of antimicrobials

A total of four antimicrobials were used in this study, including traditional and eco-friendly sanitizers (Table 4.2). The traditional sanitizers included a quaternary ammonium compound (QUAT) benzalkonium chloride (BAC; Acros Organics, Geel, Belgium) that is not commercially used, and a commercially available QUAT sanitizer (CQAC; Professional Lysol® No Rinse Sanitizer, Lysol, Parsippany, NJ). The eco-friendly sanitizers included an alcohol-based (ALB; Purell™ Food Service Surface Sanitizers, Purell, Akron, OH) and a citric acid-based sanitizer (CAB; PRO-SAN® L, Microcide Inc., Mountain View, CA).

Antimicrobials were used as-is (ALB, CAB) or prepared following manufacturer recommendations (CQAC). Since BAC is not commercially used as a sanitizer, concentrations and exposure times were based on the recommendation for other QUAT sanitizers (Table 4.2). Antimicrobials were stored at 4°C for up to one week. For QUAT sanitizers, QUAT concentrations were measured using QUAT test strips (MQuant Quaternary Ammonium Compound test strips, MilliporeSigma, Burlington, MA).

Table 4.2 Antimicrobials (n=4) tested in this study, including their active ingredients and concentrations, the manufacturer recommended usage and exposure time (MRC/ET), and the concentrations tested in minimum bactericidal concentration (MBC) determination.

Sanitizer	Active Ingredient(s)	Label MRC/ET[1]
Alcohol-based (ALB) sanitizer	Alcohol	RTU[2] /1 min[3]
Benzalkonium chloride (BAC)[4]	QUAT	200 ppm[5]/2 min
Commercial QAC (CQAC)	QUAT	200 ppm/2 min
Citric acid-based (CAB) sanitizer	Citric acid Sodium dodecylbenzene sulfonate (SDS)	RTU/1 min[6]

[1]MRC/ET, manufacturer recommended concentration and exposure time.
[2]RTU, ready-to-use solution.
[3]Active ingredient (ethyl alcohol) content in undiluted solution at 29.4% (v/v).
[4]Since benzalkonium chloride (BAC) is not a commercially used sanitizer, MRC/ET values were based on the recommendations for other QUAT sanitizers.
[5]Ppm, parts per million, equivalent to 200 μg/ml.
[6]Active ingredients in undiluted solution: citric acid content at 0.660% (w/v%); SDS at 0.036% (w/v%).

4.2.3 Attachment of isolates to common food contact surface materials and exposure to antimicrobials

Single colonies of each *L. monocytogenes* strain were transferred from BAP plates into 5 ml of TSB, followed by incubation at 37°C for 16±2 h, with shaking (150 rpm; Thermo Scientific, MaxQ4000, Waltham, MA). Cultures were diluted to approximately 5 log CFU/ml in 0.1% peptone water (PW; Fisher Scientific, Hampton, NH). Inoculum was confirmed by enumeration, using the track dilution method described by Jett et al. (1997). Samples were serially diluted (1:10) in PW, plated side by side on TSA plates, and incubated at 37°C for 24 h. Sterile SS and high-density polyethylene (HDPE) plastic coupons (1.3 cm x 1.3 cm) were placed in sterile 12-well microtiter plates (VWR Tissue Culture Plates, VWR, Radnor, PA), and inoculated with 1 ml of culture (5 log CFU/ml). Isolates were allowed to attach to coupons at 4°C or 35°C for 24 h without shaking.

Coupons were removed from microtiter plates using sterile polypropylene forceps and gently rinsed with autoclaved distilled, de-ionized water (ddH_2O). Rinsed coupons were placed into sterile 12-well microtiter plates containing antimicrobials at manufacturer recommended concentrations (MRC) and exposed to sanitizer treatments according to exposure times provided on the label (MRC/ET; Table 4.2). Following the treatment, sterile forceps were used to remove coupons and placed them in tubes containing 10 ml of Dey-Engley broth (DEB; Difco Laboratories Inc., Detroit, MI). DEB tubes were incubated at 37°C for 24 h. A color change from purple to

yellow indicated the presence of viable bacteria. Results were confirmed by streaking DEB onto TSAYE plates, incubated at 37ºC for 24 h. Each treatment was run in duplicate and repeated five times.

Attachment experiments were also completed with the addition of a cleaning step prior to exposure to antimicrobials at 30°C, using the isolate that showed highest survivability in the attachment experiments without the cleaning step (i.e. WRLP-360). Following the 24 h attachment period at 30°C, as described in the paragraphs above, the SS and plastic coupons were air-dried for 5 min in a biosafety cabinet, rinsed with sterile ddH_2O, and both sides were scrubbed using sterile cotton swabs dipped in detergent (Dawn Ultra, Proctor & Gamble, Cincinnati, OH). Following cleaning, coupons were rinsed with ddH_2O and submerged in antimicrobials at MRC/ET. Following antimicrobial treatments, coupons were transferred to DEB tubes. DEB tubes were incubated at 37ºC for 24 h, and color change from purple to yellow was recorded. Results were confirmed by streaking DEB onto TSAYE plates, incubated at 37ºC for 24 h. Each treatment was run in duplicate and repeated four times.

4.2.4 Scanning electron microscopy

The exposure to BAC, CQAC, ALB and CAB antimicrobials was further investigated for isolate WRLP-360 using scanning electron microscopy (SEM). Following sanitizer treatments described in section 4.2.3, single plastic and SS from all four antimicrobials tested were removed from the DEB tubes and rinsed gently with ddH_2O. Coupons were fixed in 1% paraformaldehyde (Paraformaldehyde, 16%, Ted Pella, Inc., Redding, CA) and 2.5% glutaraldehyde (Glutaraldehyde, 505 soln., VWR, Radnor, PA) in 0.1 M cacodylate buffer (Sodium cacodylate, 0.1 M buffer soln., pH 7.0, Alfa Aesar, Haverhill, MA) at room temperature overnight, followed by soaking in 0.1 M cacodylate buffer in two 15-minute increments. Ethanol (EtOH; Anhydrous Ethyl Alcohol, 200 Proof, Absolute, Pharmco-Aaper, Shelbyville, KY) gradient (10%, 30%, 50%, 70%, 90%, 100%) was used to dehydrated coupons. First, coupons were placed in 10% EtOH for 15 min before being moved to 30% EtOH for another 15 min. This continued until coupons reached the highest EtOH concentration, at which point they were soaked twice in 100% EtOH for 15 min. Coupons were dried in a critical point drier (EMS 850 Critical Point Drier, Electron Microscopy Sciences, Hatfield, PA) and mounted onto 20 mm aluminum SEM stubs. Lastly, coupons were sputter coated with gold/palladium (Sputter Coater 108auto, Cressington Scientific Instruments,

Watford, UK). Micrographs of 3-5 locations on the top surface of each coupon, were captured, and bacterial presence was visualized using a scanning electron microscope (Quanta 600 FEG, Thermo Fisher Scientific, Waltham, MA).

4.3 Results

4.3.1 Survival of *L. monocytogenes* attached to plastic and steel coupons

Survival of *L. monocytogenes* isolates attached to SS and plastic coupons after exposure to sanitizers at MRC/ET, and without cleaning prior to sanitizing, is shown in Table 4.3. Without the cleaning step, at 30ºC all sanitizers failed to consistently and completely inactivate *L. monocytogenes* cells. When attached to either SS or plastic at 30ºC for 24 h and treated with BAC and CAB all isolates tested had survival rates over 70% (7/10). The CQAC and ALB sanitizers had the highest inactivation frequency on SS and plastic coupons, respectively, when *Lm* attachments occurred at 30°C. However, complete inactivation with CQAC and ALB was not achieved, with three isolates (OSY-428; LR59-1; WRLP-360) and four isolates (08-5578; LR59-1; WRLP-354; WRLP-360), respectively, with survival rates 50% or higher (Table 4.3).

Table 4.3. Incidence of survival of *Listeria monocytogenes* (*Lm*) isolates (n=5) attached to stainless steel and plastic coupons and treated with an alcohol-based (ALB) sanitizer, benzalkonium chloride (BAC), a commercial quaternary ammonium compound (CQAC), and a citric acid-based (CAB) sanitizer at 4ºC and 30ºC without cleaning prior to sanitizing. Each sample was run in duplicate and experiments were completed five times. Results represent the fraction of positives (i.e. growth occurred), with bolded fractions indicating a survival rate of 50% or more.

***Lm* Isolate**	**No. Times Survived/Tested**							
	ALB		**BAC**		**CQAC**		**CAB**	
	4ºC	**30ºC**	**4ºC**	**30ºC**	**4ºC**	**30ºC**	**4ºC**	**30ºC**
Stainless Steel								
OSY-428	4/10	4/10	**9/10**	**8/10**	**7/10**	**8/10**	1/10	**8/10**
08-5578	**5/10**	**8/10**	**8/10**	**9/10**	4/10	4/10	1/10	**8/10**
LR-59-1	3/10	**5/10**	**8/10**	**10/10**	4/10	**6/10**	2/10	**9/10**
WRLP-354	3/10	**7/10**	**9/10**	**8/10**	**6/10**	4/10	3/10	**8/10**
WRLP-360	4/10	**10/10**	**9/10**	**10/10**	**8/10**	**5/10**	4/10	**10/10**
Plastic								
OSY-428	0/10	2/10	4/10	**10/10**	1/10	**8/10**	1/10	**9/10**
08-5578	0/10	**5/10**	3/10	**9/10**	1/10	**8/10**	0/10	**7/10**
LR-59-1	0/10	**6/10**	3/10	**10/10**	1/10	**9/10**	1/10	**9/10**
WRLP-354	0/10	**6/10**	2/10	**10/10**	0/10	**6/10**	1/10	**10/10**
WRLP-360	0/10	**9/10**	1/10	**9/10**	2/10	**7/10**	1/10	**10/10**

The total number of positives (i.e. presence of growth) when attachment occurred at 30ºC (308/400) was almost 2.5 times higher than the number of positives when attachment occurred at 4ºC (124/400) (Table 4.4). Increased inactivation at 4ºC was especially apparent when 4ºC-attached isolates were treated with CAB. None of the isolates survived more than 40% of the time on either SS or plastic following 4ºC attachment compared to survival rates of 70% or higher when attached at 30ºC (Table 4.3). On SS, only one 4ºC-attached isolate (08-5578) had a survival rate exceeding 50% after treatment with ALB, thought the same isolate and other isolates tested were completely inactivated by ALB on plastic (Table 4.3).

At 30ºC, survival of *L. monocytogenes* was similar on SS (149/400) and plastic (159/400) coupons (Table 4.4). However, when attachment occurred at 4ºC, the total number of positives on plastic (22/200) was over 4.6 times lower than on SS (102/200) (Table 4.4). The number of positives decreased by 68.5% and 23.5% on plastic and SS, respectively, when attachment and sanitizer treatment temperature was lowered to 4ºC (Table 4.4). While the overall number of positive results on both SS and plastic decreased when temperature was decreased to 4ºC, the effect was more prominent on plastic.

Table 4.4. Combined incidence of survival of five *L. monocytogenes* isolates following treatment with an alcohol-based (ALB) sanitizer, benzalkonium chloride (BAC), a commercial quaternary ammonium compound (CQAC), and a citric acid-based (CAB) sanitizer at 4ºC or 30ºC. Each sample was run in duplicate and experiments were completed five times. Results represent the combined total fraction and percentage of positives (i.e. growth occurred) of all isolates and treatments.

Sanitizers	**No. Times Survived/Tested (Survival%)**	
	4ºC	**30ºC**
Stainless Steel		
ALB	19/50 (38%)	34/50 (68%)
BAC	43/50 (86%)	45/50 (90%)
CQAC	29/50 (58%)	27/50 (54%)
CAB	11/50 (22%)	43/50 (86%)
Plastic		
ALB	0/50 (0%)	28/50 (56%)
BAC	13/50 (26%)	48/50 (96%)
CQAC	5/50 (10%)	38/50 (76%)
CAB	4/50 (8%)	45/50 (90%)
Total	***124/400 (31%)***	***308/400 (77%)***

4.3.2 Effect of cleaning on inactivation of *L. monocytogenes* attached to stainless steel and plastic coupons prior to sanitation

Addition of a cleaning step prior to sanitizing SS coupons did not greatly decrease the survival of WRLP-360 with any sanitizer treatment tested when bacteria were allowed to attach at 30°C for 24 h (Table 4.5). Without a cleaning step, WRLP-360 survival on SS when treated with BAC, CAB, and ALB was 100% (10/10), while treatment with CQAC resulted in a 50% survival rate (5/10) (Table 4.3). With the addition of a cleaning step, the incidence of survival decreased to 75% (6/8) when coupons were treated with ALB and CAB but remained at 100% (8/8) when treated with BAC. Incidence of survival increased for CQAC treatment (8/8) with cleaning, indicating potential irregularity in attachment or treatment itself (Table 4.5).

The addition of a cleaning step when WRLP-360 was attached to plastic resulted in similar incidence of survival for CQAC, with 6/8 and 7/10 positive samples observed with and without cleaning, respectively. Similarly, exposure of plastic coupons to BAC resulted in 8/8 and 9/10 positive rate for treatment with and without cleaning, respectively. However, the inactivation of WRLP-360 attached to plastic with eco-friendly sanitizers increased with the addition of a cleaning step. Without the cleaning step, 90% (9/10) and 100% (10/10) survival rates were observed for WRLP-360 following ALB and CAB treatments, respectively (Table 4.3). With cleaning, survival decreased to 25% (2/8) for both ALB and CAB (Table 4.5).

Table 4.5. Incidence of survival of *Listeria monocytogenes* WRLP-360 isolates when attached to stainless steel and plastic coupons for 24 h following scrubbing with detergent and treatment with an alcohol-based (ALB) sanitizer, benzalkonium chloride (BAC), a commercial quaternary ammonium compound (CQAC), and a citric acid-based (CAB) sanitizer at 30°C. Each sample was run in duplicate and experiments were repeated four times. Results represent the fraction of positives (i.e. growth occurred), with bolded fractions indicating a survival rate of 50% or more.

	No. Times Survived/Tested	
Sanitizer	**Stainless Steel**	**Plastic**
ALB	**6/8**	2/8
BAC	**8/8**	**8/8**
CQAC	**8/8**	**6/8**
CAB	**6/8**	2/8

4.3.3 Scanning electron microscopy analysis

When coupons inoculated with WRLP-360 were visualized using scanning electron microscopy (SEM), micrographs confirmed that positive controls, consisting of inoculated

coupons not treated with sanitizers, from 30ºC attachment had overall more *L. monocytogenes* attached to surfaces compared to coupons with a 4ºC attachment temperature (Fig. 4.1). Additionally, SEM revealed that the SS coupons had more variation on the surface than plastic coupons, with more divots and scratches observed on SS coupons. While survival of some isolates during treatments with or without the cleaning step was observed, comparison of the positive controls (i.e. samples not treated with sanitizers) to treated samples, showed that all sanitizers tested were able to decrease overall population of *L. monocytogenes* on SS and plastic surfaces (Fig. 4.1 and Fig. 4.2).

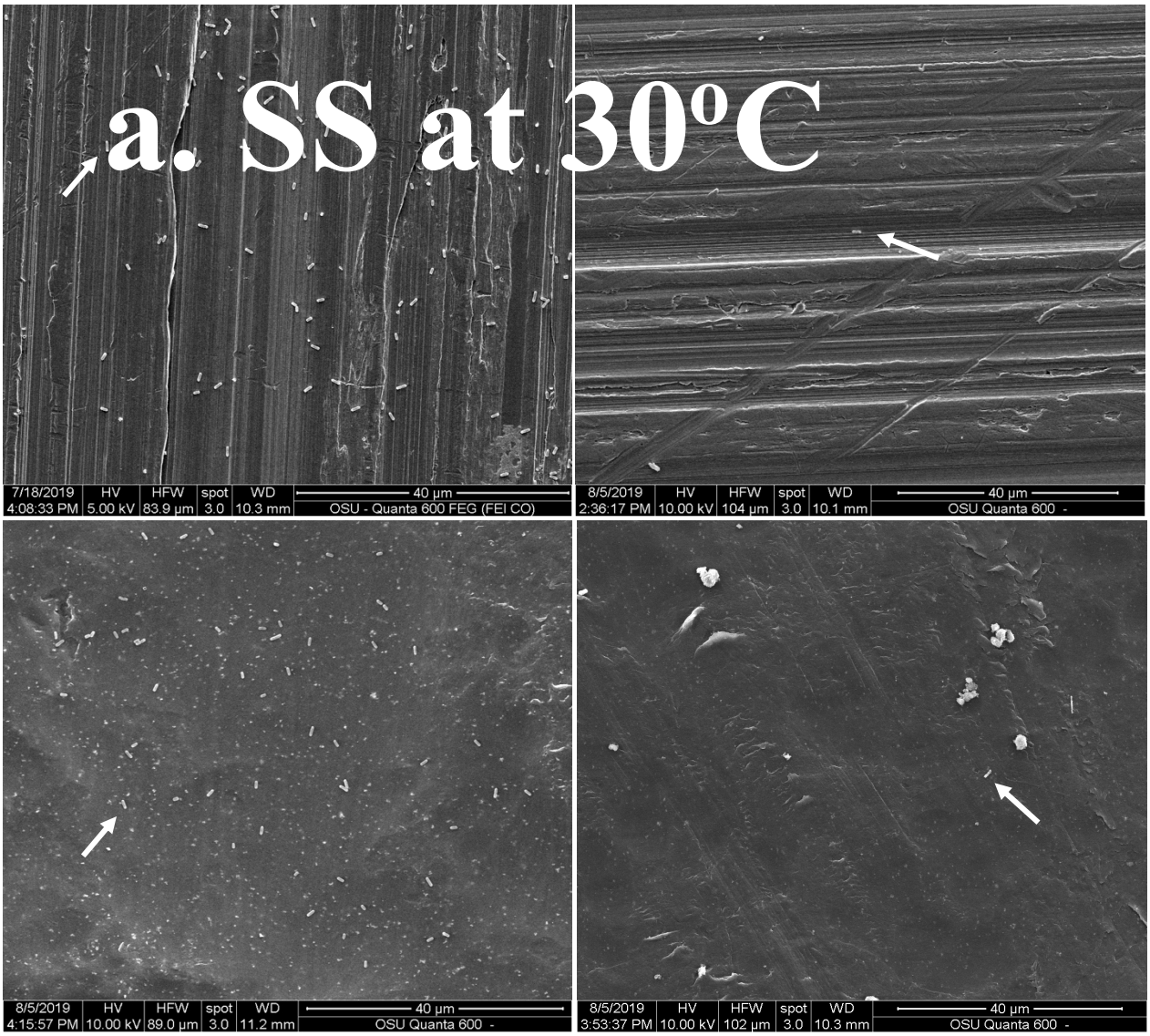

Figure 4.1. Scanning electron micrographs of *Listeria monocytogenes* isolate WRLP-360 attached to (a) stainless steel (SS) at 30ºC, (b) SS at 4ºC, (c) plastic at 30ºC, and (d) plastic at 4ºC for 24 h. Arrows are indicating to *L. monocytogenes* cells.

Despite the positive results obtained, all sanitizers included in this study were able to decrease the population of *L. monocytogenes* present as determined by visual comparison of micrographs from positive controls to those treated with sanitizer (Fig. 4.1 and Fig. 4.2).

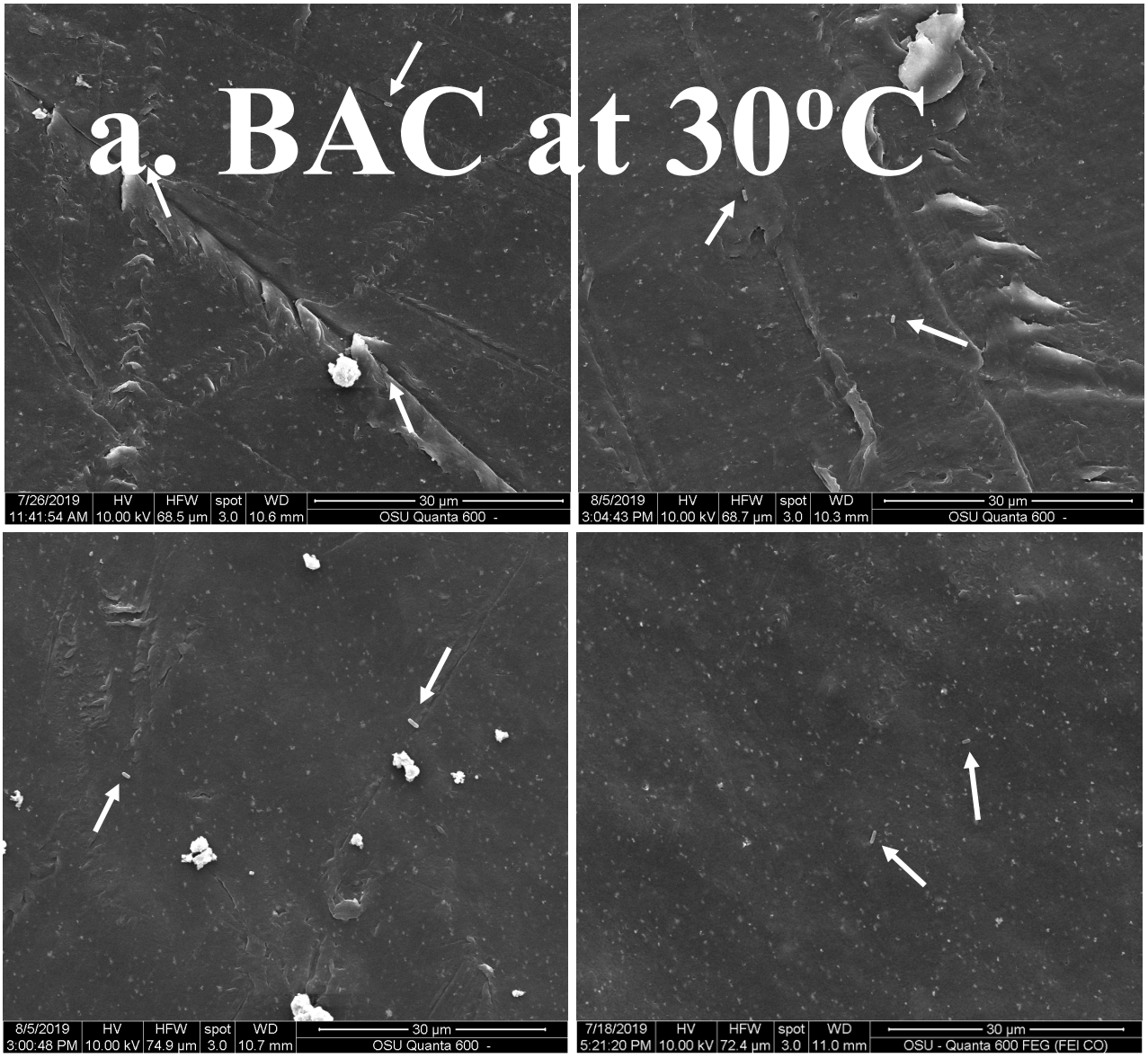

Figure 4.2. Scanning electron micrographs of plastic coupons following attachment of *Listeria monocytogenes* isolate WRLP-360 at 30ºC for 24 h and exposed to (a) benzalkonium chloride (BAC) and (b) a commercial quaternary ammonium compound (CQAC), (c) an alcohol-based (ALB), and (d) a citric acid-based (CAB) sanitizer at their manufacturer recommended concentrations and exposure times. Arrows are indicating to *L. monocytogenes.*

4.4 Discussion

The ability of sanitizers to control *L. monocytogenes* isolates (n=5) was severely impaired when bacteria were allowed to attach to SS and plastic coupons at 30°C for 24 h. All sanitizers tested failed to completely inactivate bacteria under these conditions when they were applied at MRC/ET. Previously determined MBCs of these sanitizers against the five isolates tested showed that sanitizers were able to control 5 log CFU/ml of planktonic bacteria at concentrations significantly lower than the MRC (Boucher et al., 2019). The same sanitizers applied at the same temperature were less effective against the isolates when they were allowed to attach to SS or plastic, indicating that the observed decrease in susceptibility was due to the 24 h attachment period. This is in agreement with other studies that demonstrated increased tolerance to sanitizers and other bactericides in sessile bacteria (Davies, 2003; Frank and Koffi, 1990; Krysinski et al., 1992).

Sanitizer efficacy improved when bacteria were attached at 4°C, with the overall incidence of survival at 31%. However, with the exception of ALB, sanitizers were still unable to consistently and completely inactivate all bacteria attached to plastic at 4°C (Table 4.3). The increased efficiency of sanitizers at 4°C was likely due to a decrease in the number of attached bacteria at 4°C compared to 30°C, as observed by SEM (Fig. 4.1 – 4.2). Decreased ability of *L. monocytogenes* to attach to a variety of materials, including steel and rubber, at lower temperatures has been described in earlier studies (Herald and Zottola, 1988; Mai and Conner, 2007; Smoot and Pierson, 1998).

The frequency of survival of *L. monocytogenes* was comparable between plastic and SS coupons when attachment occurred at 30°C (Table 4.4). However, when attachment occurred at 4°C, SS had a higher incidence of positive results for all sanitizers tested. With 4°C attachment, *L. monocytogenes* survival on SS was 14% to 60% higher compared to plastic, with treatment of coupons with CAB and BAC representing the lowest and highest shift in survival rate, respectively (Table 4.4). Attachment at 4°C to plastic was also associated with lower survival rates compared to those attached at 30°C (Table 4.4). Findings in earlier studies indicated that properties of the surface material can affect the rate and strength of *L. monocytogenes* attachment, including the surface hydrophobicity, free energy, and electric charge (Silva et al., 2008; Smoot and Pierson, 1998; Tuson and Weibel, 2013; Veluz et al., 2012). Differences in surface microtopography have also been found to have an effect on the ability of *L. monocytogenes* to survive sanitation, with

smoother surfaces often resulting in increased inactivation (Krysinski et al., 1992). As indicated by SEM, SS coupons were less smooth compared to the plastic, which may have partially contributed to the difference in inactivation observed here between plastic and SS coupons. Considering the similar survival rates between SS and plastic at 30°C and the dramatic drop in the survival rate from plastic at 30°C to plastic at 4°C, discrepancies in the interaction of *L. monocytogenes* with the surfaces have likely also had an effect.

The addition of a cleaning step (i.e. scrubbing of coupons with detergent) slightly reduced the incidence of survival of WRLP-360 on SS treated with ALB and CAB, but a more notable reduction in survival rate was observed when ALB and CAB were used to treat plastic coupons with WRLP-360 attached (Table 4.5). For BAC and CQAC, no increase in inactivation was observed with the addition of cleaning prior to sanitation (Table 4.5). Although *L. monocytogenes* is normally susceptible to cleaning and sanitation, instances of cleaning and sanitation regimens failing to completely inactivate surface-adhered *L. monocytogenes* has been documented, especially when bacteria are allowed to attach for prolonged times and form biofilms (Gram et al., 2007; Krysinski et al., 1992).

The results presented here indicate that *L. monocytogenes* can become more tolerant to cleaning and sanitation when given a 24 h attachment period on SS and plastic, both of which are common food contact surface materials found in food processing facilities. In facilities producing or handling RTE foods, sanitation and maintenance teams should consider adding a clean break within a 24 h period, to decrease the risk of survival of sanitizer-tolerant, surface-adherent *L. monocytogenes.* Additionally, visual monitoring and replacing of equipment and surfaces when scratches and divots form should be considered. Differences in inactivation between surface materials and exposure temperature highlight variables that are important when developing procedures and frequencies for cleaning and sanitizing regimes. Increased attachment and survival at higher temperatures indicated that facilities operating at slightly higher temperatures should be especially vigilant. Furthermore, to avoid the potential of sub-lethal sanitizer exposures and the development of *L. monocytogenes* tolerance to sanitizers that can also lead to co-selection and cross-protection against a variety of antibiotics and other stresses (Kovacevic et al., 2013; Lunden et al., 2003), it is imperative that cleaning and sanitizing protocols are effective in eliminating these microorganisms from RTE areas.

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Chapter 5: Conclusions

Since being classified as a foodborne pathogen in the 1980s, *L. monocytogenes* has been linked to outbreaks and recalls associated with products in the meat, seafood, dairy, and produce industries. As an environmental pathogen, products most often become contaminated with *L. monocytogenes* through the processing environment. Therefore, limiting the presence of *L. monocytogenes* in this area is key in reducing the risk of contamination. Cleaning and sanitation are very important and fundamental elements of *L. monocytogenes* control programs in food processing facilities. However, the ongoing occurrence of outbreaks and recalls associated with *L. monocytogenes* are evidence that current control methods are not always adequately controlling this pathogen. Additionally, increased availability of eco-friendly sanitizers for use in food processing facilities requires ascertaining the efficacy of these compounds when exposed to conditions relevant to food processing environments. The goal of this thesis was to describe how conditions encountered in food processing facilities may affect the efficacy of two traditional and two eco-friendly sanitizers to control *Listeria* spp.

Analysis of the minimum bactericidal concentrations (MBCs) of four sanitizers, benzalkonium chloride (BAC), a commercial quaternary ammonium compound (CQAC), an alcohol-based (ALB), and a citric acid-based (CAB) sanitizer, against 22 *Listeria* spp. isolates revealed that all sanitizers were able to control planktonic isolates at concentrations well below the manufacturer recommended concentrations (MRCs). With more frequent observations of antimicrobial resistance and tolerance in *L. monocytogenes* (Dutta et al., 2013; Morvan et al., 2010; Walsh et al., 2001), it was encouraging to see that these sanitizers were capable of inactivating all isolates tested. Based on the percentage of the MRC, the MBCs of traditional sanitizers were significantly lower than those of eco-friendly sanitizers ($P < 0.001$), indicating that the traditional sanitizers tested could endure more application errors while still being effective. Assessment of MBCs across temperatures ranging from 4–30^{o}C revealed that isolates were more tolerant to sanitizers at 4^{o}C as opposed to 30^{o}C. As cold temperature alters the cell membrane physiology and activates the sigma-B (σ^{B}) stress response in *L. monocytogenes*, this trend of increasing MBC values as temperature decreases is likely partially due to increased tolerance associated with innate stress mechanisms, but additional mechanisms can also be contributing to the observed stress

tolerance. These findings highlight the effect temperature has on sanitizer efficacy (Annous et al., 1997; Becker et al., 2000).

Attachment of the more tolerant *L. monocytogenes* isolates, as determined by MBCs, greatly impaired the efficacy of sanitizers. Incomplete inactivation was observed for all isolates attached to SS and plastic coupons at 30ºC following treatment with all sanitizers tested. As opposed to trends observed in MBC data, sanitizers were more effective when attachment and exposure were carried out at 4ºC compared to 30ºC. This trend can be explained by differences in the relative amount of attachment at 4ºC compared to 30ºC. Scanning electron micrographs revealed that the presence of *L. monocytogenes* on SS and plastic coupons was lower when attachment occurred at 4ºC compared to 30ºC, confirming that *L. monocytogenes* struggles more to attach at lower temperatures. While cold temperature is widely used to prolong the shelf life of food products, as *L. monocytogenes* can grow at refrigeration temperatures, even low numbers of bacteria can grow to harmful levels, especially in coolers and other cold temperature-controlled areas. These areas should still be cleaned and sanitized with regularity. The materials of equipment and other surfaces should also be taken into consideration, as differences between rate of inactivation were seen between the surface materials tested. SS resulted in a higher incidence of survival compared to plastic. While not specifically investigated in the current study, previous research has shown that the strength of attachment and the surface texture are important factors contributing to *L. monocytogenes* survival in food processing areas (Krysinski et al., 1992; Smoot and Pierson, 1998; Tuson and Weibel, 2013). To decrease the likelihood of the survival of *L. monocytogenes* on surfaces, equipment and processing areas should be kept in good condition and frequently cleaned and sanitized. An attachment time of 24 h resulted in *L. monocytogenes* resistant to cleaning and sanitation, indicating that cleaning and sanitizing may need to occur more often than once every 24 h in facilities where *Listeria* spp. are commonly encountered. Cleaning and sanitation of equipment and the processing environment before and after processing is a common practice in the industry and should aid in preventing the occurrence of surface adhered *L. monocytogenes*. However, these processes should be carried out carefully in order to avoid hard to reach areas being missed or inadequately treated, which may allow for bacterial attachment and *L. monocytogenes* exposure to sub-lethal sanitizer concentrations.

As seen in sub-lethal sanitizer exposure experiments, isolates can adapt to and grow in low levels of sanitizers. In these experiments, a range of the more and less susceptible isolates, as

determined by MBC values, were exposed to sub-lethal concentrations of sanitizers for 24 h, while their growth was monitored. Although neither were completely inactivated, two of the isolates (08-5578_Δ1862, WRLP-382) were unable to grow in BAC or CQAC over the 24 h surveillance period. One of these isolates was a Δ*emrE* mutant, missing *emrE* gene that encodes for an efflux pump associated with increased tolerance to quaternary ammonium compounds (QUATs). The other isolate was recently recovered from a produce handling environment in U.S., with its AMR profile currently being assessed. Overall, incidence of AMR genes in *L. monocytogenes* isolates recovered from produce facilities specifically has not been tracked. However, Dutta et al. (2013) reported that 70 of 116 isolates tested, originating from various sources, possessed *bcrABC*. They speculated that while this cassette is widely distributed, it does not occur universally in *L. monocytogenes* isolates. Still, the occurrence of these and other efflux pumps in *L. monocytogenes* isolates should continue to be monitored as increased tolerance to sanitizers can have severe consequences.

In summary, *L. monocytogenes* isolates that survive the stresses of food processing environments, such as exposure to sanitizers, can become more tolerant to other stresses, leading to increased challenges in controlling them in these environments (Abeysundara et al., 2019; Hecker et al., 2007; Kazmierczak et al., 2003; van Schaik and Tjakko, 2005). Additionally, exposure to low levels of sanitizers may select for tolerant phenotypes, contributing to increased prevalence of tolerant strains, as seen with the spread of antibiotic resistant bacteria (Morente et al., 2013; Romanova et al., 2006). While no isolate resistant to sanitizers at their MRC have been observed, increased tolerance in isolates combined with improper use of sanitizers or the protection afforded by cell attachment to surfaces, increase the risk of survival and persistence of isolates in food processing environments. The work presented here demonstrates that the sanitizers tested were capable of controlling a wide range of planktonic *Listeria* spp. isolates. However, variance between strains, temperature, and especially attachment affected sanitizer efficacy. Variables such as these should be considered by food processors when designing and implementing their cleaning and sanitation protocols.

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APPENDIX

Appendix
Data from minimum bactericidal concentration and sub-lethal sanitizer concentration exposure experiments

Table A.1. Minimum bactericidal concentrations (MBC; reported as % of the manufactured recommended concentration, %MRC) of an alcohol-based (ALB) sanitizer against *Listeria* spp. at temperatures ranging from 4 to 30ºC. Experiments were completed three times and *Listeria* spp. isolates (n=22) were run in duplicate. Results represent the average from three experiments, with standard error of the mean (SEM).

Isolate ID	ALB minimum bactericidal concentrations (%MRC±SEM)[1]			
	4ºC	15ºC	23ºC	30ºC
L. innocua				
ME19-1	25.0 ± 0.0a**	22.9 ± 2.1b	16.7 ± 2.6b	12.5 ± 0.0b
WRLP-357	39.6 ± 6.8a	25.0 ± 0.0ab	22.9 ± 2.1b*	12.5 ± 0.0b**
L. monocytogenes				
Scott A	29.2 ± 4.2a	25.0 ± 0.0a	25.0 ± 0.0a	12.5 ± 0.0b*
OSY-428	37.5 ± 5.6a	33.3 ± 5.3a	25.0 ± 0.0ab	12.5 ± 0.0b*
Ohio	33.3 ± 5.3a	29.2 ± 4.2a	20.8 ± 2.6ab	8.3 ± 1.3b*
ATCC 19116	41.7 ± 5.3a**	25.0 ± 0.0b	25.0 ± 0.0b	12.5 ± 0.0c*
81-0861	29.2 ± 4.2a	25.0 ± 0.0a	18.8 ± 2.8ab	12.5 ± 0.0b*
08-5578	25.0 ± 0.0a	25.0 ± 0.0a	25.0 ± 0.0a	12.5 ± 0.0b***
08-5578_1862	25.0 ± 0.0a	25.0 ± 0.0a	25.0 ± 0.0a	12.5 ± 0.0b***
FE7-1	25.0 ± 0.0a	25.0 ± 0.0a	22.9 ± 2.1a	12.5 ± 0.0b***
FE10-1	25.0 ± 0.0a	25.0 ± 0.0a	22.9 ± 2.1a	12.5 ± 0.0b***
OF28-1	25.0 ± 0.0a	25.0 ± 0.0a	22.9 ± 2.1a	12.5 ± 0.0b***
LR39-1	25.0 ± 0.0a	25.0 ± 0.0a	20.8 ± 2.6a	12.5 ± 0.0b**
LR59-1	20.8 ± 2.6a	25.0 ± 0.0a	25.0 ± 0.0a	12.5 ± 0.0b**
WRLP-354	29.2 ± 7.0a*	25.0 ± 0.0ab	25.0 ± 0.0ab	12.5 ± 0.0b*
WRLP-360	20.8 ± 2.6ab	25.0 ± 0.0a**	18.8 ± 2.8ab	12.5 ± 0.0b**
WRLP-367	25.0 ± 0.0a**	22.9 ± 2.1a*	14.6 ± 2.1b*	12.5 ± 0.0b**
WRLP-382	33.3 ± 5.3a*	25.0 ± 0.0ab	16.7 ± 2.6b*	12.5 ± 0.0b**
L. seeligeri				
DE37-2	33.3 ± 5.3a*	25.0 ± 0.0ab	25.0 ± 0.0ab	16.7 ± 2.6b*
FE37-1	29.2 ± 7.0a*	22.9 ± 2.1ab	16.7 ± 2.6ab	10.4 ± 1.3b*
L. welshimeri				
ME1-1	29.2 ± 4.2a*	22.9 ± 2.1ab	16.7 ± 2.6b*	12.5 ± 0.0b**
FF6-3	41.7 ± 5.3a*	25.0 ± 0.0b	25.0 ± 0.0b	14.6 ± 2.1b
Average MBC	29.5 ± 3.2a*	25.2 ± 0.8ab	21.7 ± 1.4b*	12.5 ± 0.3c**

[1]Values from isolates across temperatures were analyzed using one-way ANOVA and Tukey's multiple comparisons test. Different letters indicate significant differences at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

Table A.2. Minimum bactericidal concentrations (MBC; reported as % of the manufactured recommended concentration, %MRC) of benzalkonium chloride (BAC) against *Listeria* spp. at temperatures ranging from 4 to 30ºC. Experiments were completed three times and *Listeria* spp. isolates (n=22) were run in duplicate. Results represent the average from three experiments, with standard error of the mean (SEM).

Isolate ID	BAC minimum bactericidal concentrations (%MRC±SEM)[1]			
	4ºC	15ºC	23ºC	30ºC
L. innocua				
ME19-1	16.7 ± 2.6a*	10.4 ± 1.3ab	6.3 ± 0.0b**	7.3 ± 1.0b*
WRLP-357	12.5 ± 0.0a	12.5 ± 0.0a	9.4 ± 1.4a	9.4 ± 1.4a
L. monocytogenes				
Scott A	14.6 ± 2.1a	11.5 ± 1.0a	9.4 ± 1.4a	8.3 ± 1.3a
OSY-428	14.6 ± 2.1a	12.5 ± 0.0a	10.4 ± 1.3a	12.5 ± 4.0a
Ohio	12.5 ± 0.0a	12.5 ± 0.0a	10.4 ± 1.3a	7.3 ± 4.0a
ATCC 19116	12.5 ± 0.0a	11.5 ± 1.0a	7.3 ± 1.0a	11.5 ± 3.0a
81-0861	12.5 ± 0.0a*	7.3 ± 1.0b*	6.3 ± 0.0b**	9.4 ± 1.4ab
08-5578	14.6 ± 2.1a	12.5 ± 0.0a	8.3 ± 1.3a	9.4 ± 1.4a
08-5578_1862	12.5 ± 0.0a**	12.5 ± 0.0a**	6.3 ± 0.0b***	8.3 ± 1.3b**
FE7-1	12.5 ± 0.0a	14.6 ± 2.1a	10.4 ± 1.3a	14.6 ± 2.1a
FE10-1	12.5 ± 0.0a	12.5 ± 0.0a	7.3 ± 1.0b***	12.5 ± 0.0a
OF28-1	12.5 ± 0.0a*	12.5 ± 0.0a*	8.3 ± 1.3b*	8.3 ± 1.3b*
LR39-1	12.5 ± 0.0a	11.5 ± 1.0a	9.4 ± 1.4a	10.4 ± 1.3a
LR59-1	14.6 ± 2.1a*	12.5 ± 0.0ab	8.3 ± 1.3b*	12.5 ± 0.0ab
WRLP-354	12.5 ± 0.0a*	8.3 ± 1.3b	7.3 ± 1.0b	6.3 ± 0.0b
WRLP-360	12.5 ± 0.0ab	14.6 ± 2.1b*	8.3 ± 1.3a*	7.3 ± 1.0a*
WRLP-367	12.5 ± 0.0a	12.5 ± 0.0a	10.4 ± 1.3a	12.5 ± 0.0a
WRLP-382	12.5 ± 0.0a	12.5 ± 0.0a	8.3 ± 1.3a	12.5 ± 2.8a
L. seeligeri				
DE37-2	12.5 ± 0.0a	12.5 ± 0.0a	6.3 ± 0.0a	12.5 ± 4.0a
FE37-1	12.5 ± 0.0a*	11.5 ± 1.0ab	7.3 ± 1.0b*	8.3 ± 1.3ab
L. welshimeri				
ME1-1	12.5 ± 0.0a*	8.3 ± 1.3ab	7.3 ± 1.0b*	7.3 ± 1.0b*
FF6-3	12.5 ± 0.0a	11.5 ± 1.0a	9.4 ± 1.4a	8.3 ± 1.3a
Average MBC	13.1 ± 0.5a**	11.7 ± 0.7ab	8.3 ± 1.0b**	9.9 ± 1.6ab

[1]Values from isolates across temperatures were analyzed using one-way ANOVA and Tukey's multiple comparisons test. Different letters indicate significant differences at $P < 0.05$ (*) and $P < 0.001$ (***).

Table A.3. Minimum bactericidal concentrations (MBC; reported as % of the manufactured recommended concentration, %MRC) of a commercially available quaternary ammonium compound (CQAC) against *Listeria* spp. at temperatures ranging from 4 to 30ºC. Experiments were completed three times and *Listeria* spp. isolates (n=22) were run in duplicate. Results represent the average from three experiments, with standard error of the mean (SEM).

Isolate ID	CQAC minimum bactericidal concentrations (%MRC±SEM)[1]			
	4ºC	**15ºC**	**23ºC**	**30ºC**
L. innocua				
ME19-1	3.6 ± 0.5a*	3.1 ± 0.0ab	3.1 ± 0.0ab	2.1 ± 0.3b*
WRLP-357	3.1 ± 0.0a	3.1 ± 0.0a	2.9 ± 0.3a	2.6 ± 0.3a
L. monocytogenes				
Scott A	3.1 ± 0.0a	3.1 ± 0.0a	3.1 ± 0.0a	2.6 ± 0.3a
OSY-428	3.1 ± 0.0a	3.1 ± 0.0a	3.1 ± 0.0a	3.1 ± 0.0a
Ohio	3.1 ± 0.0a	3.1 ± 0.0a	3.1 ± 0.0a	0.3 ± 0.1b***
ATCC 19116	3.1 ± 0.0a	3.1 ± 0.0a	3.6 ± 0.5a	3.1 ± 0.0a
81-0861	2.9 ± 0.3a	3.1 ± 0.0a	3.1 ± 0.0a	2.9 ± 0.3a
08-5578	3.1 ± 0.0a	3.1 ± 0.0a	3.1 ± 0.0a	2.6 ± 0.3a
08-5578_1862	3.1 ± 0.0a	3.1 ± 0.0a	3.1 ± 0.0a	2.6 ± 0.3a
FE7-1	3.1 ± 0.0a	3.1 ± 0.0a	3.6 ± 0.5a	2.9 ± 0.3a
FE10-1	3.6 ± 0.5a	3.1 ± 0.0a	3.1 ± 0.0a	2.6 ± 0.3a
OF28-1	3.1 ± 0.0a	3.1 ± 0.0a	3.1 ± 0.0a	2.6 ± 0.3a
LR39-1	3.1 ± 0.0a	3.1 ± 0.0a	3.6 ± 0.5a	3.1 ± 0.7a
LR59-1	3.1 ± 0.0a	3.1 ± 0.0a	3.1 ± 0.0a	2.6 ± 0.3a
WRLP-354	3.6 ± 0.5a	3.1 ± 0.0a	2.9 ± 0.3a	2.6 ± 0.3a
WRLP-360	3.1 ± 0.0a	2.9 ± 0.3a	3.6 ± 0.5a	2.9 ± 0.3a
WRLP-367	3.1 ± 0.0a	3.1 ± 0.0a	2.9 ± 0.3a	2.9 ± 0.3a
WRLP-382	3.6 ± 0.5a	3.1 ± 0.0a	3.1 ± 0.0a	2.6 ± 0.3a
L. seeligeri				
DE37-2	3.1 ± 0.0a	3.6 ± 0.5a	3.6 ± 0.5a	2.6 ± 0.3a
FE37-1	3.1 ± 0.0a	3.1 ± 0.0a	2.6 ± 0.3a	2.6 ± 0.3a
L. welshimeri				
ME1-1	3.1 ± 0.0a*	3.1 ± 0.0a*	2.6 ± 0.3ab	1.8 ± 0.3b*
FF6-3	4.2 ± 0.7a	3.1 ± 0.0a	3.1 ± 0.0a	2.6 ± 0.3a
Average MBC	3.3 ± 0.1a*	3.1 ± 0.0ab	3.2 ± 0.2ab	2.3 ± 0.3b*

[1]Values from isolates across temperatures were analyzed using one-way ANOVA and Tukey's multiple comparisons test. Different letters indicate significant differences at $P < 0.05$ (*) and $P < 0.001$ (***).

Table A.4. Minimum bactericidal concentrations (MBC; reported as % of the manufactured recommended concentration, %MRC) of a citric acid-based (CAB) sanitizer against *Listeria* spp. at temperatures ranging from 4 to 30ºC. Experiments were completed three times and *Listeria* spp. isolates (n=22) were run in duplicate. Results represent the average from three experiments, with standard error of the mean (SEM).

Isolate ID	CAB minimum bactericidal concentrations (%MRC±SEM)[1]			
	4ºC	15ºC	23ºC	30ºC
L. innocua				
ME19-1	25.0 ± 0.0a**	14.6 ± 2.1b	12.5 ± 0.0b	14.6 ± 2.1b
WRLP-357	37.5 ± 5.6a*	33.3 ± 5.3ab	16.7 ± 2.6b*	22.9 ± 2.1ab
L. monocytogenes				
Scott A	16.7 ± 2.6a	12.5 ± 0.0a	12.5 ± 0.0a	12.5 ± 0.0a
OSY-428	50.0 ± 0.0a***	18.8 ± 6.3b	12.5 ± 0.0b	12.5 ± 0.0b
Ohio	4.7 ± 0.7a	5.2 ± 0.7a	5.7 ± 0.5a	1.2 ± 0.4b*
ATCC 19116	31.3 ± 6.3a	22.9 ± 6.0a	14.6 ± 2.1a	16.7 ± 2.6a
81-0861	31.3 ± 6.3a	25.0 ± 0.0a	22.9 ± 2.1a	25.0 ± 0.0a
08-5578	41.7 ± 5.3a*	18.8 ± 6.3b*	20.8 ± 2.6b*	25.0 ± 0.0ab
08-5578_1862	41.7 ± 5.3a*	20.8 ± 6.2b*	12.5 ± 0.0b*	29.2 ± 4.2ab
FE7-1	45.8 ± 4.2a**	12.5 ± 0.0b	12.5 ± 0.0b	20.8 ± 6.2b
FE10-1	50.0 ± 0.0a**	18.8 ± 6.3b*	14.6 ± 2.1b**	41.7 ± 5.3a*
OF28-1	41.7 ± 5.3a*	18.8 ± 2.8b	12.5 ± 0.0b	22.9 ± 2.1b
LR39-1	45.8 ± 4.2a*	37.5 ± 5.6ab	20.8 ± 2.6b*	33.3 ± 5.3ab
LR59-1	50.0 ± 0.0a	41.7 ± 5.3a	43.8 ± 6.3a	43.8 ± 6.3a
WRLP-354	41.7 ± 5.3a*	18.8 ± 6.3ab	12.5 ± 0.0b*	33.3 ± 7.7ab
WRLP-360	45.8 ± 4.2a***	12.5 ± 0.0b	14.6 ± 2.1b	16.7 ± 2.6b
WRLP-367	31.3 ± 6.3a*	14.6 ± 2.1b*	12.5 ± 0.0b*	20.8 ± 2.6ab
WRLP-382	37.5 ± 5.6a	33.3 ± 7.7a	25.0 ± 5.6a	41.7 ± 5.3a
L. seeligeri				
DE37-2	50.0 ± 0.0a	37.5 ± 7.9a	29.2 ± 9.5a	25.0 ± 0.0a
FE37-1	50.0 ± 0.0a***	50.0 ± 0.0a***	12.5 ± 0.0b***	18.8 ± 6.3b***
L. welshimeri				
ME1-1	50.0 ± 0.0a***	50.0 ± 0.0a***	22.9 ± 2.1b***	25.0 ± 0.0b***
FF6-3	41.7 ± 5.3ab*	50.0 ± 0.0a*	29.2 ± 4.2bc*	25.0 ± 0.0c*
Average MBC	39.1 ± 3.3a*	25.8 ± 3.5b	17.9 ± 2.0b	24.0 ± 2.8b

[1]Values from isolates across temperatures were analyzed using one-way ANOVA and Tukey's multiple comparisons test. Different letters indicate significant differences at $P < 0.05$ (*) and $P < 0.001$ (***).

Table A.5. Average minimum bactericidal concentrations (MBCs; μg/ml) of benzalkonium chloride (BAC) and a commercially available quaternary ammonium compound (CQAC) at temperatures ranging from 4 to 30°C against *Listeria* spp. (n=22). Experiments were performed in duplicate for each isolate and repeated three times. Results represent the average MBCs and standard error of the mean (SEM) of all isolates from three independent experiments.

Temperature	Average minimum bactericidal concentration (μg/ml ± SEM)[1]	
	BAC	CQAC
4°C	26.14 ± 1.00a***	6.51 ± 0.27b***
15°C	23.48 ± 1.31a***	6.27 ± 0.07b***
23°C	16.57 ± 2.06a**	6.32 ± 0.37b**
30°C	19.70 ± 3.18a**	5.12 ± 0.58b**

[1]Values were analyzed using two-way t-tests to determine significant differences between sanitizers across different temperatures at $P < 0.01$ (**) and $P < 0.001$ (***).

Table A.6. Average minimum bactericidal concentrations (MBCs) of an alcohol-based (ALB) sanitizer, benzalkonium chloride (BAC), a commercially available quaternary ammonium compound (CQAC), and a citric acid-based (CAB) sanitizer at temperatures ranging from 4 to 30°C presented as % manufacturer recommended concentration (%MRC) against *Listeria* spp. (n=22). Experiments were performed in duplicate for each isolate and repeated three times. Results represent the average MBCs and standard error of the mean (SEM) of all isolates from three independent experiments.

Sanitizer	Average minimum bactericidal concentration (%MRC ± SEM)[1]			
	4°C	15°C	23°C	30°C
ALB	29.50 ± 3.20a*[2]	25.20 ± 0.80a[2]	21.70 ± 1.4a[2]	12.50 ± 0.30a[3]
BAC	13.10 ± 0.50b*	11.70 ± 0.70b**	8.30 ± 1.00b*	9.85 ± 1.60a
CQAC	3.30 ± 0.10c*	3.10 ± 0.00c**	3.20 ± 0.20c*	2.56 ± 0.29b*
CAB	39.10 ± 3.30d*	25.80 ± 3.50a	17.90 ± 2.00a	24.00 ± 2.80c***

[1]Values from sanitizers were analyzed across temperatures using ANOVA and Tukey's multiple comparisons test (vertical comparison only). Different letters indicate significant differences at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

[2]*P* values between eco-friendly sanitizers (ALB and CAB) and traditional sanitizers (BAC and CQAC) were significant at $P < 0.001$ (***).

[3]*P* values between eco-friendly sanitizers (ALB and CAB) and CQAC were significant at $P < 0.001$ (***).

Table A.7. Average minimum bactericidal concentrations (MBCs) of an alcohol-based (ALB) sanitizer, benzalkonium chloride (BAC), a commercially available quaternary ammonium compound (CQAC), and a citric acid-based (CAB) sanitizer at temperatures ranging from 4 to 30°C presented as % manufacturer recommended concentration (%MRC) against *L. monocytogenes* (*Lm*) isolates with sanitizer antimicrobial resistance genes (S-AMR; n=6) and *Listeria* spp. isolates where presence of S-AMR genes is unknown (Unk; n=16). Experiments were performed in duplicate for each isolate and repeated three times. Results represent the average MBCs and standard error of the mean (SEM) of all isolates from three independent experiments.

	MBC (%MRC± SEM)[1]			
***Lm* Isolates**	**ALB**	**BAC**	**CQAC**	**CAB**
4°C				
S-AMR	24.3 ± 0.7a	13.2 ± 0.4a	3.2 ± 0.1a	45.8 ± 1.5a
Unknown S-AMR	**31.4 ± 1.6b***	13.0 ± 0.3a	3.3 ± 0.1a	**36.6 ± 3.2b*****
15°C				
S-AMR	25.0 ± 0.0a	12.7 ± 0.4a	3.1 ± 0.0a	24.7 ± 4.9a
Unknown S-AMR	25.3 ± 0.7a	11.7 ± 0.4a	3.1 ± 0.0a	26.3 ± 3.9a
23°C				
S-AMR	23.3 ± 0.6a	8.7 ± 0.4a	3.3 ± 0.1a	20.8 ± 4.8a
Unknown S-AMR	21.1 ± 1.0a	8.1 ± 0.4a	3.1 ± 0.1a	16.8 ± 1.7a
30°C				
S-AMR	12.5 ± 0.0a	11.3 ± 0.9a	2.7 ± 0.1a	31.3 ± 4.0a
Unknown S-AMR	12.5 ± 0.4a	9.3 ± 0.6a	2.5 ± 0.2a	21.3 ± 2.4a

[1]Values from S-AMR and Unk were analyzed at each temperature using unpaired t-tests with Welch's correction (vertical comparison within each temperature only). Different letters (in bold) indicate significant differences at $P < 0.05$ (*) and $P < 0.01$ (**).

Table A.8. Average minimum bactericidal concentrations (MBCs) of an alcohol-based (ALB) sanitizer, benzalkonium chloride (BAC), a commercially available quaternary ammonium compound (CQAC), and a citric acid-based (CAB) sanitizer at temperatures ranging from 4 to 30ºC presented as % manufacturer recommended concentration (%MRC) against *L. monocytogenes* (*Lm*) isolates 08-5578_Δ1862 (Δ*emrE*) and 08-5578 (wt). Experiments were performed in duplicate for each isolate and repeated three times. Results represent the average MBCs and standard error of the mean (SEM) of all isolates from three independent experiments.

	MBC (%MRC± SEM)[1]			
***Lm* Isolates**	**ALB**	**BAC**	**CQAC**	**CAB**
4ºC				
08-5578_Δ1862	25.0 ± 0.7a	14.6 ± 2.1a	3.1 ± 0.0a	41.7 ± 5.3a
08-5578	25.0 ± 0.0a	12.5 ± 0.0a	3.1 ± 0.0a	41.7 ± 5.3a
15ºC				
08-5578_Δ1862	25.0 ± 0.0a	12.5 ± 0.0a	3.1 ± 0.0a	18.8 ± 6.3a
08-5578	25.0 ± 0.0a	12.5 ± 0.0a	3.1 ± 0.0a	20.8 ± 6.2a
23ºC				
08-5578_Δ1862	25.0 ± 0.0a	6.3 ± 0.0a	3.1 ± 0.0a	12.5 ± 0.0a
08-5578	25.0 ± 0.0a	8.3 ± 1.3a	3.1 ± 0.1a	20.8 ± 2.6a
30ºC				
08-5578_Δ1862	12.5 ± 0.0a	8.3 ± 1.3a	2.6 ± 0.3a	29.2 ± 4.2a
08-5578	12.5 ± 0.0a	9.4 ± 1.4a	2.6 ± 0.3a	25.0 ± 0.0a

[1]Values from 08-5578_Δ1862 and 08-5578 were analyzed at each temperature using unpaired t-test with Welch's correction (vertical comparison within each temperature only). No significant differences were found at $P < 0.05$.

Table A.9. Growth of *L. monocytogenes* (*Lm*) isolates (n=8) in trypticase soy broth with sub-lethal concentrations of an alcohol-based (ALB; 6.25% manufacturer recommended concentrations [MRC]) sanitizer at 30°C. The data represent the average log CFU/ml (± standard deviation [SD]) values of three independent assays.

***Lm* isolate**	**Average log CFU/ml ± SD[1]**						
	0 h	**1 h**	**3 h**	**5.5 h**	**8 h**	**10 h**	**24 h**
OSY-428	5.69 ± 0.21ab	5.69 ± 0.15a	5.53 ± 0.22a	5.57 ± 0.26a	5.53 ± 0.33a	5.51 ± 0.18a	6.27 ± 0.40a
08-5578	5.63 ± 0.18ab	5.73 ± 0.32a	5.66 ± 0.37a	5.52 ± 0.24a	5.55 ± 0.08a	5.6 ± 0.33a	5.91 ± 0.06a
08-5578 _Δ1862	5.77 ± 0.23ab	5.67 ± 0.32a	5.62 ± 0.23a	5.61 ± 0.29a	5.56 ± 0.25a	5.57 ± 0.40a	6.05 ± 0.11a
FE7-1	5.62 ± 0.30ab	5.49 ± 0.19a	5.57 ± 0.24a	5.12 ± 0.59a	5.85 ± 0.57a	5.49 ± 0.25a	**5.05 ± 0.05b****
FE10-1	**5.48 ± 0.16a***	5.49 ± 0.28a	5.44 ± 0.26a	5.42 ± 0.28a	5.50 ± 0.32a	5.48 ± 0.23a	**5.23 ± 0.18b***
LR39-1	5.73 ± 0.26ab	5.66 ± 0.29a	5.68 ± 0.38a	5.60 ± 0.26a	5.48 ± 0.39a	5.69 ± 0.32a	5.96 ± 0.31a
LR59-1	5.74 ± 0.37ab	5.51 ± 0.12a	5.37 ± 0.35a	5.60 ± 0.35a	5.59 ± 0.23a	5.59 ± 0.29a	6.00 ± 0.23a
WRLP-382	**5.86 ± 0.20b***	5.66 ± 0.24a	5.71 ± 0.27a	5.65 ± 0.28a	5.64 ± 0.35a	5.72 ± 0.36a	6.25 ± 0.14a

[1]Log CFU/ml counts from each time point were compared among isolates using one-way ANOVA with Tukey's multiple comparison test. Statistically significant values are represented with different letters (in bold) at $P < 0.05$ (*) and $P <. 0.01$ (**).

Table A.10. Growth of *L. monocytogenes* (*Lm*) isolates (n=8) in in trypticase soy broth with sub-lethal concentrations of benzalkonium chloride (BAC; 6.25 μg/ml) at 30°C. The data represent the average log CFU/ml (± standard deviation [SD]) values of three independent assays.

***Lm* isolate**	**Average log CFU ± SD[1]**						
	0 h	**1 h**	**3 h**	**5.5 h**	**8 h**	**10 h**	**24 h**
OSY-428	5.61 ± 0.23a	5.46 ± 0.27a	5.20 ± 0.39a	5.17 ± 0.73a	5.26 ± 0.99a	5.31 ± 1.30a	**7.69 ± 1.20a***
08-5578	5.75 ± 0.39a	5.46 ± 0.22a	5.44 ± 0.26a	5.33 ± 0.33a	5.18 ± 0.50a	4.98 ± 0.67a	5.62 ± 0.15ab
08-5578 _Δ1862	5.69 ± 0.25a	5.51 ± 0.25a	5.58 ± 0.31a	5.42 ± 0.17a	5.12 ± 0.19a	4.87 ± 0.29a	**3.75 ± 0.84b***
FE7-1	5.62 ± 0.25a	5.51 ± 0.37a	5.32 ± 0.24a	5.39 ± 0.63a	5.20 ± 0.79a	5.35 ± 0.98a	7.14 ± 1.47ab
FE10-1	5.50 ± 0.33a	5.39 ± 0.18a	5.37 ± 0.55a	5.48 ± 0.47a	5.37 ± 0.52a	5.25 ± 0.47a	7.12 ± 1.80ab
LR39-1	5.69 ± 0.36a	5.50 ± 0.18a	5.44 ± 0.30a	5.30 ± 0.34a	5.29 ± 0.43a	5.14 ± 0.54a	6.09 ± 1.17ab
LR59-1	5.67 ± 0.25a	5.48 ± 0.26a	5.41 ± 0.59a	5.48 ± 0.59a	5.52 ± 0.97a	5.52 ± 1.04a	7.19 ± 1.87ab
WRLP-382	5.62 ± 0.14a	5.56 ± 0.35a	5.41 ± 0.38a	5.18 ± 0.15a	4.75 ± 0.57a	4.66 ± 0.65a	3.80 ± 1.13ab

[1]Log CFU/ml counts from each time point were compared among isolates using one-way ANOVA with Tukey's multiple comparison test. Statistically significant values are represented with different letters (in bold) at $P < 0.05$ (*).

Table A.11. Growth of *L. monocytogenes* (*Lm*) isolates (n=8) in trypticase soy broth with sub-lethal concentrations of a commercial quaternary ammonium compound (CQAC; 1.56 μg/ml) at 30°C. The data represent the average log CFU/ml (± standard deviation [SD]) values of three independent assays.

***Lm* isolate**	**Average log CFU ± SD[1]**						
	0 h	**1 h**	**3 h**	**5.5 h**	**8 h**	**10 h**	**24 h**
OSY-428	5.57 ± 0.35a	5.35 ± 0.39a	5.35 ± 0.37a	5.29 ± 0.23a	5.29 ± 0.36ab	5.37 ± 0.32a	7.39 ± 0.28a
08-5578	5.64 ± 0.23a	5.43 ± 0.29a	5.45 ± 0.23a	5.40 ± 0.24a	**4.95 ± 0.27a***	4.90 ± 0.31a	6.42 ± 0.96a
08-5578 _Δ1862	5.63 ± 0.31a	5.57 ± 0.29a	5.44 ± 0.36a	5.21 ± 0.18a	5.20 ± 0.29ab	5.10 ± 0.16a	**3.48 ± 0.45b*****
FE7-1	5.57 ± 0.34a	5.48 ± 0.13a	5.41 ± 0.30a	5.26 ± 0.05a	5.11 ± 0.33ab	5.29 ± 0.36a	6.80 ± 0.58a
FE10-1	5.61 ± 0.18a	5.41 ± 0.25a	5.29 ± 0.31a	5.29 ± 0.19a	5.27 ± 0.09ab	5.13 ± 0.31a	6.64 ± 0.43a
LR39-1	5.56 ± 0.12a	5.57 ± 0.24a	5.57 ± 0.34a	5.38 ± 0.26a	5.29 ± 0.26ab	5.12 ± 0.17a	6.60 ± 0.65a
LR59-1	5.74 ± 0.30a	5.54 ± 0.23a	5.38 ± 0.35a	5.27 ± 0.35a	5.08 ± 0.59ab	5.17 ± 0.41a	6.80 ± 0.50a
WRLP-382	5.67 ± 0.22a	5.53 ± 0.22a	5.28 ± 0.15a	5.24 ± 0.54a	**4.53 ± 0.21b***	**4.25 ± 0.28b***	3.48

[1]Log CFU/ml counts from each time point were compared among isolates using one-way ANOVA with Tukey's multiple comparison test. Statistically significant values are represented with different letters (in bold) at $P < 0.05$ (*) and $P < 0.001$ (***).

Table A.12. Growth of *L. monocytogenes* (*Lm*) isolates (n=8) in trypticase soy broth with sub-lethal concentrations of a citric acid-based (CAB; 6.25% manufacturer recommended concentrations [MRC]) sanitizer at 30°C. The data represent the average log CFU/ml (± standard deviation [SD]) values of three independent assays.

***Lm* isolate**	**Average log CFU ± SD[1]**						
	0 h	**1 h**	**3 h**	**5.5 h**	**8 h**	**10 h**	**24 h**
OSY-428	5.52 ± 0.12a	5.54 ± 0.37a	5.40 ± 0.39a	5.74 ± 0.40a	6.26 ± 0.35a	6.63 ± 0.35a	8.90 ± 0.16ab
08-5578	5.56 ± 0.30a	5.53 ± 0.31a	5.65 ± 0.37a	5.79 ± 0.38a	6.17 ± 0.36a	6.34 ± 0.44a	8.34 ± 0.39ab
08-5578 _Δ1862	5.69 ± 0.29a	5.48 ± 0.15a	5.63 ± 0.36a	5.88 ± 0.48a	6.11 ± 0.52a	6.21 ± 0.57a	**8.22 ± 0.57a***
FE7-1	5.64 ± 0.45a	5.52 ± 0.23a	5.69 ± 0.43a	5.75 ± 0.38a	6.15 ± 0.44a	6.37 ± 0.54a	8.27 ± 0.36ab
FE10-1	5.52 ± 0.29a	5.53 ± 0.29a	5.46 ± 0.33a	5.64 ± 0.46a	6.01 ± 0.56a	6.21 ± 0.56a	**8.13 ± 0.16a***
LR39-1	5.63 ± 0.23a	5.57 ± 0.25a	5.60 ± 0.24a	5.77 ± 0.20a	6.32 ± 0.50a	6.43 ± 0.54a	8.60 ± 0.05ab
LR59-1	5.61 ± 0.36a	5.51 ± 0.20a	5.57 ± 0.35a	5.87 ± 0.46a	6.49 ± 0.31a	7.10 ± 0.85a	8.91 ± 0.04ab
WRLP-382	5.71 ± 0.30a	5.72 ± 0.31a	5.85 ± 0.29a	6.02 ± 0.80a	7.30 ± 0.72a	7.32 ± 0.49a	**9.11 ± 0.22b***

[1]Log CFU/ml counts from each time point were compared among isolates using one-way ANOVA with Tukey's multiple comparison test. Statistically significant values are represented with different letters (in bold) at $P < 0.05$.

Table A.13. Growth of *L. monocytogenes* (*Lm*) isolates (n=8) in trypticase soy broth at 30°C. The data represent the average log CFU/ml (± standard deviation [SD]) values of three independent assays.

***Lm* isolate**	**Average log CFU ± SD[1]**						
	0 h	**1 h**	**3 h**	**5.5 h**	**8 h**	**10 h**	**24 h**
OSY-428	5.50 ± 0.13a	5.75 ± 0.25a	6.60 ± 0.38a	7.53 ± 0.23a	8.41 ± 0.17a	9.02 ± 0.15a	9.35 ± 0.08a
08-5578	5.58 ± 0.41a	5.77 ± 0.29a	6.51 ± 0.18a	7.50 ± 0.19a	7.63 ± 1.56a	8.12 ± 1.76a	9.45 ± 0.10a
08-5578 _Δ1862	5.60 ± 0.29a	5.82 ± 0.19a	6.65 ± 0.46a	7.45 ± 0.23a	8.44 ± 0.24a	9.07 ± 0.20a	9.39 ± 0.07a
FE7-1	5.51 ± 0.20a	5.70 ± 0.38a	6.59 ± 0.39a	7.51 ± 0.38a	8.55 ± 0.32a	8.97 ± 0.14a	9.48 ± 0.10a
FE10-1	5.58 ± 0.40a	5.72 ± 0.28a	6.54 ± 0.30a	7.44 ± 0.23a	8.42 ± 0.24a	8.86 ± 0.27a	9.27 ± 0.07a
LR39-1	5.62 ± 0.25a	5.79 ± 0.30a	6.53 ± 0.29a	7.53 ± 0.25a	8.36 ± 0.06a	9.01 ± 0.26a	9.38 ± 0.06a
LR59-1	5.70 ± 0.35a	5.81 ± 0.35a	6.45 ± 0.39a	7.50 ± 0.27a	8.51 ± 0.22a	9.03 ± 0.19a	9.35 ± 0.06a
WRLP-382	5.70 ± 0.40a	5.82 ± 6.52a	6.52 ± 0.20a	7.52 ± 0.28a	8.40 ± 0.31a	9.09 ± 0.25a	9.47 ± 0.10a

[1]Log CFU/ml counts from each time point were compared among isolates using one-way ANOVA with Tukey's multiple comparison test. No statistically significant differences were found at $P < 0.05$.