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Biofilm Sanitizer Tolerance of Vermont Dairy Listeria monocytogenes

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BIOFILM SANITIZER TOLERANCE OF VERMONT DAIRY *LISTERIA MONOCYTOGENES*

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ABSTRACT

Listeria monocytogenes is a foodborne pathogen found in biofilms on surfaces and equipment in the food processing environment. Sodium hypochlorite (SH) and quaternary ammonium compounds (QAC) are readily available and commonly used sanitizers. However, due to the structure and additional organic material produced in a biofilm, killing bacteria within the biofilm may be a challenge for one or both of these sanitizers.

The objective of this work was to determine if immature and mature biofilms from *L. monocytogenes* isolated from Vermont artisan dairy environments were more tolerant to QAC and SH compared to planktonic cultures' tolerance. To determine sanitizer minimum inhibitory concentration (MIC), cultures were incubated statically in 1x or 1/20x Brain Heart Infusion broth (BHI) in polystyrene microtiter plates for 24 hours at room temperature (22°C) with serial dilutions of sanitizer. Sanitizer efficacy on biofilms was determined by growing isolates on one cm stainless steel coupons in 1x or 1/20x BHI to simulate nutrient rich and poor conditions on a surface commonly seen in food processing environments. Coupons were incubated statically for 1, 3, or 10 days. Media was replaced every 48 hours to prevent nutrient depletion. After incubation, coupons were rinsed 3 times with phosphate buffered saline and placed into 0, 50, 100, or 200ppm SH or QAC for the manufacturer recommended contact time. Sanitizer was neutralized and adherent cells were removed by vortexing with beads. Cell suspensions were diluted and plated, then counted via spot plating on BHI agar. Significant differences for biofilm survival were assessed using Analysis of Variance in R (v.4.0.4).

MICs for isolates grown in nutrient poor (1/20X BHI) conditions were lower than nutrient rich conditions (1X BHI) for both sanitizers. All isolates' biofilms reached ~6-8 log10 CFU/coupon on stainless steel. Reductions from different QAC concentrations differed (p_{adj} <0.05) in 1/20x BHI but were not significantly different in 1x BHI. In both biofilm growth conditions SH was more effective at 200 ppm than 50 ppm (p_{adj} < 0.05). Biofilms from both persistent and transient *L. monocytogenes* environmental isolates from Vermont dairies are resistant to working concentrations of QAC sanitizer, but sodium hypochlorite bleach more adequately reduces *L. monocytogenes* biofilm on stainless steel.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1: Literature Review

Listeria monocytogenes

Listeria monocytogenes is a gram-positive bacterium commonly transmitted through food (1). It is a facultative anaerobe, and it can survive under a pH range of 4.1-9.6, which encompasses a wide range of foods (2). *L. monocytogenes* is also capable of survival and growth at temperatures ranging from 0-45°C, which makes it of particular concern as a foodborne pathogen in products which are stored under refrigeration conditions (2). It is usually found in foods such as fresh produce and ready-to-eat meat (3), and it can even be found in more unexpected places such as caramel apples or ice cream (4, 5). Ingestion of these bacteria can lead to serious illness, known as listeriosis (6). The threat of contracting listeriosis is highest to pregnant, elderly, young, and immunocompromised people (1). In its most devastating presentations it can cause septicemia, meningitis, and spontaneous abortion (1).

In the United States, there are an estimated 1,591 listeriosis cases annually with a hospitalization rate of 94%, and death rate of 15.9% (7). The number of bacteria required to make someone ill is variable (8). In the case of the Blue Bell ice cream outbreak, contamination was estimated to be an average 8 most probable number per gram (8). Ten people in nursing homes were hospitalized and three died as a result of this outbreak, which also suggests that other factors such as age, medication, and immune system can play into more severe listeriosis illnesses (1, 2). These preventable foodborne illnesses and deaths can be avoided by adhering to simple things like hygiene, routine cleaning and disinfection, and other good manufacturing practices in food production (9); if these

opportunities are missed, *L. monocytogenes* can reproduce during the product's shelf life, even in refrigerated foods, and potentially harm consumers (3).

L. monocytogenes in Dairy

Dairy foods like milk, cheese, and yogurt have all the necessary prerequisites for bacteria to thrive, including protein, fat, water availability, and near-neutral pH (10). Consequently, *L. monocytogenes* is a pathogen of major concern in dairy, particularly in unpasteurized raw milk and cheese, as this bacterium can be present in soil, water, or raw ingredients and contaminate a product at virtually any time during processing (1, 3). Cows' milk itself can be a source of *Listeria spp*., or uncleaned feces and soil from the cow's udder during milking may contaminate the milk with bacteria (1, 8). Raw milk and cheese are of greater concern than pasteurized dairy products, as there is no kill step present; due to an increased risk of pathogen contamination, many regulations surround both production and consumption (11). Pasteurization will kill any *L. monocytogenes* present in the raw milk (10); however, pasteurization alone is not enough to protect from this pathogen (10). *L. monocytogenes* can be brought into a production environment in many other ways such as with employees, on packaging, or in other ingredients and be a source of post-processing contamination (10).

No matter how a facility becomes contaminated, *L. monocytogenes* may establish itself and persist in ecological niches in the food processing environment due to incomplete or inadequate cleaning procedures (9). In a study that looked at how *L. monocytogenes* could detach from stainless steel surfaces and contaminate dairy products, researchers observed that pathogens initially grown on coupons immersed in

yogurt or milk had increased growth rates when transferred to fresh milk (12). This demonstrated the risk of detachment and contamination in a dairy environment and showed how dairy-adapted biofilms have an advantage in milk and can grow to higher levels more quickly, posing a risk to product (12).

L. monocytogenes in Conventional Dairy

The dairy industry in the United States is dominated by conventional commercial dairies. These are large farms with potentially hundreds of head of milking cattle, which produce milk to be pasteurized, packaged and processed in factories off-site (13). Pasteurization is the heat treatment of milk to kill pathogens, and it is required in the US for milk that is sold over state lines (14). This kill step is very important for milk safety and quality when it is processed, but conventional and commercial dairies can still have issues with *L. monocytogenes* (10). If pasteurizers break down or are not working at full capacity as milk is pumped through, there can be incomplete pasteurization which leaves dangerous microbes behind (10). Post-processing contamination is also a possibility if there are niches in a facility where *L. monocytogenes* is present (10).

L. monocytogenes is an ongoing concern for conventional and artisan dairies. In 2002 and 2007, *L. monocytogenes* was isolated from 6.5% and 7.1% respectively of bulk milk samples prior to pasteurization (15, 16). Jackson et al. observed a higher prevalence in commingled silo milk, with 12.5% positive samples for *L. monocytogenes*, but that may be due to detection and sampling methods used, and levels of *Listeria spp*. were low overall (17). In Vermont, specifically for milk used in artisan cheese making, a study found that there was no *L. monocytogenes* in any of the 101 milk samples collected from

21 farmstead operations (18). While *L. monocytogenes* was not found in any milk, researchers have found this pathogen in Vermont dairy plants, identifying areas of particular concern like non-food contact surfaces, floors, or sanitizing foot baths that may be avenues for cross-contamination (19).

L. monocytogenes strains isolated from commercial dairy environments have been previously studied; genetic profiling reveals that dairy-isolated *L. monocytogenes* in the U.S. come from 56 clonal complexes, and may contain multiple virulence markers that are associated with increased pathogenicity in humans (20). Additionally, a study done in France revealed that there was a strong association between *L. monocytogenes* dairy isolates and clonal complexes that are considered hypervirulent (21). They hypothesized this was due to their most likely source, the gut of a cow, which they would be well-adapted to and which is similar enough to human guts to result in increased pathogenicity (21). However, the distribution of clones worldwide varies, with different clones being found more frequently in certain regions overall, so this may not be applicable to *L. monocytogenes* in the United States (22).

Artisan Dairy and *L. monocytogenes*

Over the course of the past few years, milk production nationwide in the U.S. has faced many challenges such as lower prices, competitive marketplaces, and reduction in number of licensed herds (23). For example, in the state of Vermont as of 2019 fluid milk prices had been in a 4-year decline (24). In the face of such a hardship, dairy producers continually turn to value-added products like cheese. This both preserves surfeit milk and earns a higher profit than milk (25). The transition from milk to cheese is a complicated

one, with many steps involved that may not come naturally to producers who are new to cheese making (25). For instance, facilities that are available may not have been built to the hygienic standards needed for manufacturing a food product (25). Additionally, there are different risks and considerations when using raw versus pasteurized milk, and for different types of cheeses (25).

To assess these risks, D'Amico at al. ran a study on the microbiological quality of milk used in Vermont artisan dairy production (18). They did not detect any of the targeted pathogens, including *Salmonella spp., Staphylococcus aureus, Escherichia coli* O157:H7, or *Listeria monocytogenes* (18). This shows that Vermont cheese milk is of very high microbiological quality with low levels of contamination which is ideal for artisan farmstead cheese production (18). However, simply by starting with milk of a high quality does not ensure that the final product of cheese will be completely safe and free of pathogens. Bacteria are everywhere, and pathogens may harbor in environmental niches and cross-contaminate the milk, tools, or final product at any point during production if good manufacturing practices are not followed (18). Researchers from this same lab group also conducted environmental sampling surveys in Vermont artisan cheese production environments (26). Through these surveys they found both *Listeria spp*., which are harmless to humans in most cases, as well as the human pathogen *Listeria monocytogenes* (26). This demonstrates that even though the milk being used in these facilities had no target pathogens found, the food production environment is never sterile and rarely completely free from pathogens (26).

When *L. monocytogenes* is found in floor drains or on squeegees, there is far less of a risk than when it is found in finished product, but the presence of these bacteria

offers a risk of product cross-contamination. In the root cause analysis for the Blue Bell Ice Cream outbreak, it was discovered that the contamination originated from condensation on pipes that passed over the conveyor belt (27). *L. monocytogenes* that was established in the environment contaminated the condensate that fell into uncovered ice cream containers, and caused multiple people to become ill (4, 27). This example shows that environmental Listeria poses a real threat in a food production environment. Thus, producers should use environmental monitoring to find and eliminate pathogens as well as other good manufacturing practices.

Consumer Interest in Artisan Dairy

There has been a relatively recent shift in the types of food that people are interested in purchasing and consuming away from food mass produced in factories in favor of food that is locally grown, traceable, and made in small batches by artisans or farmers (28). Cirne et al. examined the difference in physical qualities of artisanally and commercially produced cheese, ice cream, coffee, chocolate, and grain; they also surveyed consumer attitudes in relation to these products (28). Survey responses from consumers in Philadelphia and New York showed that consumers desire local and artisanal products for practical aspects such as sensory quality or nutrition, but also for more indirect values they assign to these products like ethos and social context (28). In Vermont specifically, people are very aware of artisan cheesemaking, and it becomes a point of pride for producers and consumers (29). Work done by Lahne and Trubek demonstrated that native Vermonters had positive sensory experiences surrounding locally produced artisan cheeses, which were informed not only by their physical experience consuming that food

but the social context as well (29). People are drawn to these products, despite drawbacks like higher cost or reduced accessibility. Some are further attracted by knowing the "farm story" of a cheese, feeling more of a personal connection to this food they know has been made by hand by a member of the community rather than a giant metal machine halfway across the country (29). In summary, consumers are very interested in consuming artisan dairy. But there have been several outbreaks in the recent past which have demonstrated the risks to artisanal cheese (30–32).

Relevant Outbreaks

Artisan cheeses have been implicated in recent outbreaks and recalls due to *Listeria monocytogenes* contamination. One such outbreak in 2017 that had devastating impacts was from Vulto Creamery in Walton, NY (32). In this circumstance, there was a recall of all raw milk soft cheeses produced by the facility because of final product contamination and 8 reported illnesses (32). Consumers fell ill from eating the Vulto cheese in 4 states, and two of these people died as a result of their listeriosis infections (32). *L. monocytogenes* was found on the brushes used to wash the rinds of these cheeses and was transferred through multiple batches, which led to the wide scale recall (32). Brushing rinds is an example of a riskier cheese production practice, but it is necessary for producing the desired outcome in this type of artisan cheese. Work was recently done examining potential recommendations for best practices when artisans use cheese brushes (33), which will hopefully lead to a reduction in risk for this type of cheese in the future.

Another recent event involving artisan cheeses and *L. monocytogenes* was at Consider Bardwell Farm in West Pawlett, VT in 2019 (30). This producer identified

pathogen contamination through routine finished product testing and initiated a product recall of multiple artisan cheeses, both hard and soft, that were thought to be a health risk to consumers (34). There were fortunately no illnesses linked to these products (30). However many people may not experience severe listeriosis symptoms or may even be completely asymptomatic, and therefore would never be tested to link back to the source (1).

Most recently, there was an outbreak declared in February 2021 linked to a smaller scale cheese producer in New Jersey (35). There were 13 illnesses identified and one reported death, where clinical isolates match the outbreak strain from the product (31). The cheeses involved in this outbreak are fresh soft cheeses, including queso fresco, quesillo, and requeson. They have short shelf lives, but are riskier than other cheeses because of the way that they are produced (36). They are not aged to facilitate pathogen die-off as in other varieties of cheese (37, 38) and also contain relatively high moisture and low salt content that allow Listeria to reproduce more easily (39).

L. monocytogenes Typing, Tracking, and Traceability

As molecular methods have advanced over recent years, pathogen typing in research and for tracking programs has shifted significantly. PulseNet is a pathogen tracking system used by the United States, run by the Centers for Disease Control (40). For many years they used a method called pulsed field gel electrophoresis, or PFGE (40). This method starts with DNA from a food or clinical bacterial isolate (40). Restriction enzymes are added which cut the DNA in sequence-specific places across the genome; these cutting locations will vary between different isolates of the same bacterial species depending on

their lineage, serovar, or type, as well as random mutations (40). The DNA fragments are then run on a gel, where smaller fragments travel more quickly in the electric field. The final gel gives investigators the pathogen's unique "fingerprint," which can be compared to known isolate types to give that bacterium its identity (40). Once the pathogen has been typed, it can be cross-referenced to known clinical or food outbreak types in the same database (40). When there is a pattern match, it is presumed that these isolates are related, are a part of the same cluster, and have the same origin (40). For example, if there are three people who all have the same type of *Escherichia coli* isolated from their stool and it is also the same type as was isolated from a petting zoo, this can be paired with epidemiological data and investigators can come to the conclusion that those people became ill from that petting zoo (40).

Another method used to differentiate bacteria is Ribotyping. This method is similar to PFGE in that there is a restriction enzyme that cuts DNA at a known location of the genome, which is then run in a gel (41). This step is followed by incubation in a probe that will hybridize to the sections of DNA encoding for rRNA, which makes up the ribosomes, hence the name Ribotyping (41). The probe can then be visualized to get a distinct pattern, called the ribotype. This method can be done with the RiboPrinter®, which increases reproducibility and ease of identification by streamlining a complicated multi-step process in one machine (42). Though Ribotyping is convenient, the method has been shown to be less capable of accurately discriminating between *L. monocytogenes* isolates compared to PFGE (43, 44).

Currently, the PulseNet system in the US uses whole genome sequencing to track and trace *Listeria monocytogenes* (45). All foodborne pathogens are now monitored using

core genome multilocus sequence typing (cgMLST) or whole genome multilocus sequence typing (wgMLST), which both utilize specific portions of the bacterial genome to identify the strain type (46). Whole genome sequencing (WGS) identifies the entire sequence of a bacteria's genome, not just fragments as would be identified during PCR. This is superior to PFGE or ribotyping because while those methods can identify patterns in the DNA in terms of size, there is no way to know the actual content of that DNA fragment in a gel without sequencing (47). By sequencing a subset of genes or the whole genome, you have a greater chance of correctly identifying it; you also may get information on what virulence genes, antibiotic resistance genes, or plasmids are present that may give an infectious advantage (47). Additionally, with WGS you can determine if two isolates that have the same PFGE pattern or ribotype are actually the same, or simply very closely related (47). A possible measure for degree of relatedness of organisms when using WGS is SNP count. SNP stands for single nucleotide polymorphisms, and by counting these you can determine how many bases in the DNA have changed between the two sequences (48). The greater the number of SNPs, the more distantly related you will assume the organisms are, and if they are just a few SNPs different, it is reasonable to assume they are incredibly closely related (48).

Using this more advanced WGS technology has allowed public health professionals to do a better job at identifying *L. monocytogenes* outbreaks. They are able to identify outbreaks sooner when there are fewer cases, thereby limiting the further spread of illness and numbers of illnesses overall (45). This technology can identify even smaller outbreaks than ever before, with the possibility of linking just two cases to a common source, where before those incredibly small outbreaks may have been isolated

as sporadic cases with no common link (45). The combination of identifying more outbreaks as a whole and limiting the number of cases per outbreak makes this an incredibly effective tool, and as laboratories across the states are able to afford this technology the CDC and PulseNet will be moving forward using it to track other pathogens as well (40, 45).

Persistence vs. Transience: Definitions and Pragmatic Thinking

Persistence is a concept that is often discussed in reference to pathogens isolated from food production environments (49). It is ideally meant to describe bacterial pathogens that establish themselves in a harborage in a production facility where they can evade cleaning, and therefore have the ability to continuously negatively affect the safety and quality of the food being produced. For the amount this concept is used, however, its definition can vary considerably. This variation is generally based on study parameters, such as how often researchers are able to sample in the same site in the same store over time (49). Persistence definitions in different situations will usually depend on re-isolation, either multiple isolations from multiple locations in a facility or in the same location of a facility over time. Definitions can be specific; for example Assisi et al. defined persistence as isolation during 3 months out of an available 6 month sampling period (50). They may be left vague as well, like Borucki et al. defining persistence simply as "repeated isolation" from the milk supplier (51). The definition of persistence will also likely specify how these isolates are verified with molecular methods, some of which are more reliable than others. Depending on the resolution of investigators' typing methods, there may be stricter cutoffs for identifying the 'same' strain in multiple places.

Additionally, even if the same strain is identified, there may be some issue with labeling it persistent in the sense of a sessile community that remains in a facility. If a strain is identified multiple times, it is true that it may have never been removed completely from the facility; there is also the possibility that the same bacteria are being re-introduced periodically from the same supplier over time, the same employees over time, or it could simply be a common strain in the area that frequently shows up due to multiple reasons (52). For example, in work done by Vazquez-Villanueva et al., a single PFGE type of *Listeria ivanovii* subsp. *ivanovii* was isolated 46 times and was the dominant pulsotype in the study (53). In this situation, asymptomatic animals shed the bacteria in their milk, which resulted in repeated reintroduction that may have masked true persistence (53). These reasons may cast some doubt as to whether true 'persistence' exists, and it can make studies with few or no subsequent re-samplings difficult to reconcile. Despite the difficulties in defining persistence, it is often used as a signifier to differentiate between groups of environmental isolates.

Hypothesized Persistence Mechanisms

There are several hypotheses on the reason why bacteria persist in food processing facilities, and two mechanisms that may link to increased persistence are biofilm formation and sanitizer tolerance (21, 51). A predominating theory is that strains which have an increased ability to attach and form biofilms will be more likely to become persistent, and therefore any strains that are identified as persistent in sampling surveys are more likely to have increased biofilm forming abilities in lab experiments. Sanitizer tolerance has also been hypothesized to lead to persistence, as it follows that if bacteria

are able to evade sanitizers at all, they will remain in a plant and establish themselves, thus becoming persistent. Many studies have examined these hypothesized persistence mechanisms; however, they had varying results.

External environmental variables are thought by many researchers to be the most significant factor in the persistence of *L. monocytogenes* in food processing environments. One set of investigators examining biofilm formation ability and sanitizer tolerance of food isolates found no significant correlation between these capabilities and persistence, and conjectured that persistence is due to a complex interplay of environmental factors (54). Similarly, a set of environmental *L. monocytogenes* isolates from delis has no link between phenotypic sanitizer tolerance ability and corresponding sanitizer genes (50). Finally, work done on the effect of different carbon sources on *L. monocytogenes* growth revealed that isolates grown with lactose as a carbon source were the most resistant to acid and heat stress, and also had the highest biofilm formation capacity (55). This suggests that if there is lactose available in a food production environment, such as from milk in a dairy facility, the bacteria will have an advantage simply due to the substrate available (55). While these results suggest that *L. monocytogenes* persistent and transient isolates may have no innate differences, other studies have seen significant differences in the cultures they examined.

Some *L. monocytogenes* have physiological advantages that enable them to be more efficient at invading host cells and causing disease. For example, Internalin A is a protein in the outer membrane of the cell that is used to invade host cells and more efficiently cause disease (56). Research has identified that a premature stop codon in the gene encoding this protein, *inlA*, will lead to a deficient or delayed invasion mechanism,

reducing the risk of illness to the host (57). The existence of genetically proven mechanisms for virulence suggests that there might be a genetic advantage that some strains have over others that will make them better at persisting in food processing environments. Tiong and Muriana identified genes that are involved in surface adhesion, the predecessor to biofilm formation, and suggested that these resulting proteins may be targeted by sanitation methods to reduce biofilm survival in food processing (58). Other researchers have confirmed that genes *rsbU* and *rmlA* influence the formation of biofilms in *L. monocytogenes*, and a change as small as one base difference between isolates could have an impact on their differential abilities to form biofilm (59). A group of Canadian researchers linked stress survival islet 1 presence and increased biofilm formation ability to improved persistence in serogroup 1/2a isolates (60). Additionally, some have observed increased sanitizer tolerance in persistent strains compared to transient strains; however, both sub-culturing or repeated exposure may reduce sanitizer susceptibility even in sporadic isolates (61, 62).

Biofilm Formation

A biofilm is a community of bacterial cells that are attached to a substrate, whether that is an abiotic surface or another bacterium (63). Cells in a biofilm also produce extracellular polymeric substances (EPS) to form a protective matrix, and display a change in phenotype compared to planktonic cells (63). Biofilm formation occurs in a series of steps. The first step is reversible attachment, wherein planktonic cells come into contact with a clean surface due to motility or random interactions and then remain on that surface because of Van der Waal forces (64). These cells are very easily removed, as they

are primarily resting on said surface, with no attachments produced yet (64). After reversible attachment is irreversible attachment, where the cells can sense they are on a surface and then start to produce EPS to 'stick' themselves in place (63). The next stage in biofilm formation is microcolony formation (63). The bacteria on this surface are reproducing to form colonies, but they do not yet cover the surface completely or have as much three-dimensional structure as we would expect in a developed biofilm (63). Once microcolonies reproduce to the point that they run into each other and cover the surface that is being colonized more completely, the biofilm reaches the next stage of maturation. The maturation step comes with the biofilm forming more three-dimensional structure, and at this point it becomes very difficult to disrupt (65). At the peak of maturation comes the final step of dispersion, where the biofilm has reached such size that large chunks of it may shear off with force or cells within the biofilm may disperse from the matrix on their own to find new surfaces to colonize (64). Bacteria that form a biofilm exhibit an altered phenotype as well, due to a change in gene expression, which allows them to work together collectively in this community differently than how they would as singular planktonic cells in suspension (63).

The EPS matrix is one of the key features of biofilm that separates it from planktonic cells and allows the structure to develop while also forming a barrier against environmental stressors, such as desiccation, temperature, and pH, or sanitizers. The matrix that surrounds the cells in a biofilm can contain many components, such as protein, free DNA, and polysaccharides (66–69). For *L. monocytogenes* monospecies biofilms, the matrix is primarily made up of a polysaccharide, techoic acid (67). This substance is also a component of the cell wall and has been shown to be identical in both

locations (67). The mechanism for the techoic acid becoming a part of the matrix is unknown, and no secretion mechanism has been demonstrated (67). Instead, its presence may be due to remnants of dead cells remaining behind as part of the biofilm (67).

Within this extracellular matrix, there is generally more than one bacterial species present. Studies that have been done on biofilms as they naturally occur in food processing environments show they are made up of many species of bacteria. For instance, Liu et al. analyzed the composition of floor drain biofilms which were positive for this *L. monocytogenes* (70). The study utilized molecular methods to determine the dominant bacterial species in 8 *L. monocytogenes* positive drain samples from two factories that produced fish sauce, and hoisin and oyster sauce, respectively (70). Where metagenomic read depth allowed, bacterial genera and species were identified; no samples had enough *L. monocytogenes* to be detected via metagenomic profiling (70). Researchers found that in these *L. monocytogenes*-positive samples the predominant bacterial genera were *Klebsiella spp*. and *Pseudomonas spp*., and species identified were *Pseudomonas psychrophila, Klebsiella oxytoca*, and *Aeromonas hydrophila* (70). These bacteria may interact with *L. monocytogenes* in different ways, with the potential to outcompete it or facilitate its harborage (70). Another study shows that *Ralstonia insidiosa* increased biofilm formation of major foodborne pathogens including multiple strains of *L. monocytogenes* (71). Interactions between different bacterial species can give an advantage to *L. monocytogenes* that it would not have growing by itself.

L. monocytogenes as a Biofilm Former

When it comes to biofilm formation, certain bacteria are more capable than others. For example, *Pseudomonas* spp. are well known to form robust biofilms, which leads to problems like increased drug resistance or biofilm-induced chronic infection (72). *L. monocytogenes*, in comparison, has been observed to be a weak to moderate biofilm former at best in laboratory studies (73, 74). Though it is not a particularly vigorous biofilm former, it is still capable, and it can certainly still be isolated from biofilms in food production environments (70).

Because of its importance as a foodborne pathogen, biofilm formation has been studied from many perspectives in *L. monocytogenes*. Some researchers focus on the role of specific genes, and have found evidence of important players like transcriptional regulators *sigB* and *prfA* which are essential for biofilm formation (75, 76). Others have examined the structure of *L. monocytogenes* biofilms with methods like microscopic imaging, finding that the predominant structure of isolates from multiple different locations is a "honeycomb-like morphotype" (77). *L. monocytogenes* biofilms are not composed of many layers and are not particularly thick, but they are notable for their ability to remain on surfaces despite uneven structure (78).

Other work focuses on the interactions of *L. monocytogenes* and established biofilm, and the ability of the pathogen to join a preexisting community. It has been demonstrated that *L. monocytogenes* can colonize pre-formed *Pseudomonas fluorescens* biofilms, and in those established communities grow to higher levels compared to those that *L. monocytogenes* can reach on its own (79). Not all established biofilms are equal, though, and work done investigating the variability of *L. monocytogenes* attachment

discovered that all biofilm forming *Lactococcus lactis* mutants reduce attachment compared to a clean surface (80). However, resident biofilms with a porous structure type formed by chain-making mutants had an increased level of pathogen colonization (80). Ultimately, biofilm formation is complex in itself and becomes even more complicated when considering the variables brought on by additional species in a community.

Models Used to Study Biofilm Formation

One of the most common methods to compare biofilm forming ability of different isolates is the standard crystal violet assay (51, 81). This is a helpful assay because it is high-throughput, so many isolates can be studied in a shorter amount of time, and it may be comparable to other assays with the added benefit of convenience (81). Additionally, this assay uses an objective measure, optical density, so it is easier to compare between studies (51). This method has drawbacks, though. For example, the microtiter plates that work for this type of assay come in a limited number of materials, which may not be the best proxy for the system being studied depending on the food processing area researchers are focused on. Also, in micro wells such as are on these plates for these assays, there is a limited amount of nutrients available to the bacteria forming a biofilm. This can limit the duration of the study, only giving a short snapshot of the biofilm formation and life cycle. Models utilizing surfaces suspended in static systems like test tubes and petri dishes, or even flow models such as a chemostat, can more closely emulate realistic conditions, but they are typically more time and resource intensive and therefore not advantageous for screening multiple isolates in a short period of time (82–84).

Previous studies have used many types of models to examine phenotypic biofilm formation of *L. monocytogenes* under controlled conditions (83, 85, 86). There are monospecies biofilm models and multispecies models, and growth systems can have continuous nutrient availability, limited nutrients, or somewhere in between (73, 83, 86, 87). Also, depending on the type of system being studied, researchers may incubate the biofilms at different temperatures, grow them on different surfaces, or in different types of media (88–90). Some researchers even create conditions to expose biofilms to cleaning and sanitizing multiple times, to further simulate processing protocols (52, 91). There are benefits and drawbacks to any model, and no model will ever completely approximate the true conditions a biofilm faces in a food processing environment. It is difficult to compare directly between every model, but investigators can routinely draw conclusions from their system that may be useful in learning a little more about biofilm formation and behavior in practical application. Experiments looking at the phenotypic ability of *L. monocytogenes* to form biofilms can reveal certain things about these systems, however understanding the genetic basis for biofilm formation may be key to controlling unwanted growth.

Genetics of Biofilm Formation

There is evidence that certain *L. monocytogenes* strains contain genetic elements that may put them at survival advantage in stressful situations. The role of stress survival islet 1 (SSI-1) in increased biofilm formation was first identified by Keeney et al. (92). It contains five genes linked to adaptability and survival under strenuous conditions, and presence of SSI-1 was positively correlated to the strongest biofilms (92). Additionally,

stress survival islet 2 (SSI-2) offers protection against oxidative and alkaline stresses, and benefits strains that possess it in food processing environments (93).

Difficulty in identifying whether persistent strains have increased biofilm formation or genetic markers of biofilm formation can possibly be attributed to two things. Firstly, these genetic elements and *L. monocytogenes* strains that are generally better biofilm formers may simply be very rare in the population overall. There are many more clones and communities of *L. monocytogenes* than scientists are able to sample and sequence, so where these outstanding performers do exist, they may number so low in the population we do not always detect them. Secondly, the issue of defining persistence may be muddying the results of studies. Because there is no standard definition for persistence and there is variability between studies, some isolates which are noted as persistent may in fact not be. This makes it challenging to draw conclusions about whether or not there is a true difference between the categories of persistent and transient isolates. Overall, biofilm formation is likely a factor that contributes to persistence, but further research is needed to determine if variability in biofilm formation has an impact on persistence ability.

Sanitizers Commonly Used in Dairy & Processing

Chemical sanitizers in food processing are an essential part of producing safe, quality, and consistent product (94). Different sanitizer types contain different active compounds, such as acid, iodine, peroxides, chlorine, and quaternary ammonium compounds (95); these all have unique benefits and drawbacks to their use (96). This leads to certain

sanitizers being more commonly used in certain situations, such as on food contact surfaces, in fermented foods, and in packaging disinfection.

There are only a few sanitizers that are commonly used in dairy processing due to the challenges dairy presents to sanitizers. Fluid milk has minerals, fat, and proteins; these can all affect sanitizer efficacy, and all must be considered when choosing a sanitizer (97). Additionally, when choosing sanitizers for use in a value-added dairy application, producers must consider if they will leave behind residues that will counteract the purpose of any starter culture. Finally, the different categories of sanitizers will have different potential applications in a dairy plant because of interactions with equipment material or food products themselves (98). As a result, two sanitizers that are commonly used in dairy production are quaternary ammonium compounds and sodium hypochlorite (96).

Quaternary Ammonium Compounds

Quaternary ammonium compounds (QACs) are a class of chemical sanitizers that are used frequently in a range of different applications (99). They are useful in the food production environment on food contact and other surfaces, and often used in dairy processing (96). These compounds will remain active over a period of time and not degrade as quickly as other sanitizers, but this can be detrimental if producers are working with cultured products (96). They also have the potential to leave residue in milk that could result in off flavors if the concentration of sanitizer is too high or it is inappropriately applied (100). QACs are stable sanitizers, remaining active at a wide pH range (96). They are cationic, which allows them to disrupt cell membranes and cause

cell leakage, lysis, and death (99). With their bulky structure that embeds itself in phospholipid membranes, the structural integrity of the cytoplasmic membrane is compromised to the point of destruction of bacterial cells (99). QACs are also effective against yeasts and certain lipid-containing viruses (99). Additionally, different types of QACs have different structures, and have different abilities to penetrate the membrane of different bacterial species (101).

Some bacteria have a tolerance mechanism to these compounds. Efflux pumps can remove low concentrations of QACs from inside the cell, preventing further disruption (101), but these structures are not enough to provide true resistance to the bacteria. This means that at a high enough concentration, regardless of the presence of an efflux pump, QACs will eventually kill the cell. Although some groups have observed increases in minimum inhibitory concentrations for *L. monocytogenes* strains, these are still below the concentrations of sanitizer used at working strength (99, 102). Despite these differences, QACs are still useful in controlling pathogens in the food production environment.

Sodium Hypochlorite

Sodium hypochlorite, commonly known as bleach, is another commonly used, easy to access sanitizer that can be very useful in the food production environment (94). It is a strong oxidizer, so it is reactive to a host of cellular components including membranes, proteins, and DNA (94). Its oxidizing ability make it a useful cleaner as well as sanitizer, but in the presence of heavy soil and organic matter the effectiveness is greatly reduced (103), because sodium hypochlorite degrades upon reaction. This property is useful in

that bleach decomposes to inactive compounds, and thus will be less likely to affect the food being produced (100), however, this can also be seen as a drawback, because the action of the sanitizer will not continue past its initial reaction, and if that reaction is with soil rather than pathogens, sanitization is not effective (95). Bleach is also sensitive to heat, light, and pH, so while it may be an ideal sanitizer in some circumstances, it may be rendered useless in others (103). Finally, it can also damage equipment by corroding it, and be irritating to those who are working with it (96). When using bleach in food processing, chlorine concentration is verified to be sure that the working solution will be effective for its intended use and solutions can be re-made throughout the day as needed (103).

Sanitizer Tolerance

Sanitizers are used daily in food processing environments to kill bacteria and pathogens that may be present and reduce safety and quality of products. However, despite frequent cleaning and sanitizing, pathogens are often found in food processing environments. One possible explanation for this is that the bacteria that remain after cleaning are tolerant to whatever sanitizer is being applied. While this is concerning due to the fact that bacteria may potentially be left behind as future contaminants, sanitizer tolerance should not be confused with resistance (104). Resistance is when a bacterium is able to survive in a concentration of a substance, like an antibiotic, that would normally kill it (104). Tolerance is a better representation of the phenomenon of pathogens requiring a higher than usual concentration of sanitizer for disinfection. While strains adapt to survive in

lower than lethal concentrations, they are not completely immune to these compounds (105).

Sanitizer tolerance has been studied extensively in *L. monocytogenes*. Researchers have found that certain strains of *L. monocytogenes* contain different genetic mechanisms that promote tolerance to quaternary ammonium compounds (61, 75, 106). Some studies have also observed *L. monocytogenes* QAC tolerance, but failed to find any known genetic link to this adaptive ability (107). It has also been seen that benzalkonium chloride, a common QAC, can induce a viable but not culturable state in *L. monocytogenes*, which can lead to flaws in detection and pathogen survival in food processing (108). Sodium hypochlorite application to *L. monocytogenes* can stress cells and increase lag time (109), but generally this sanitizer is the most effective when compared to others (110, 111).

Evidence for Biofilm Formation and Sanitizer Tolerance as Persistence Mechanisms

The true reason for *L. monocytogenes* persistence has proven to be challenging to pinpoint. Sanitizer tolerance or attachment and biofilm formation are attractive explanations, and an inherent genomic link to increased risk may be possible. Some studies have found a clear link between persistent strains and biofilm formation or sanitizer tolerance; other studies have found no link whatsoever between these phenotypic capabilities and persistent strains, and many did not examine the possible community interactions that may influence persistence. Consequently, persistence and transience may be arbitrary, and separate from natural abilities inherent to bacterial strains.

Some studies have found that there is a linked difference to persistence and increased biofilm forming ability (112–115). Researchers in Japan studying persistent and transient *L. monocytogenes* isolated from cold-smoked fish processing observed that the persistent strain produced relatively higher amounts of biofilm, as well as an increased amount of EPS (112). A study focusing on raw milk and non-dairy food isolates discovered that persistent strains exhibited increased adherence over a 48 hour period compared to sporadic strains, and in addition they saw that serotype 1/2c was significantly better at adhering compared to both 4b and 1/2a (113). In a study focused on *L. monocytogenes* isolated from mussel production, investigators observed that at 30°C persistent strains showed higher but not significantly greater biofilm formation compared to transient strains (114). This study also looked at biofilm formation at 20°C, where it was observed that both persistent and transient strains formed low levels of biofilm and therefore concluded that biofilm formation was likely linked to persistence in some capacity, but that what makes an isolate persistent is multifactorial and complex (114). A study done on isolates from chicken samples at a Tokyo market observed something similar, and saw that at 37°C the amount of biofilm formation by persistent strains was consistently higher than transient strains, but at 30°C there was no difference observed between the groups; investigators suggest that persistent strains have the ability to alter biofilm formation as a response to temperature and other environmental factors (115). Other studies have linked persistence to sanitizer tolerance. For example, a study done on isolates from a pig slaughterhouse found the *bcrABC* cassette which contains resistance genes to benzalkonium chloride, a common QAC, in persistent strains (116). Other genomic links to persistence could be stress survival islet 1 (SSI-1), observed by Upham

et al. to be significantly linked to increased biofilm formation and serotype 1/2a, which could give this marker potential as something to look for in risk assessments (60).

However, other studies have shown that there is no difference between persistent and transient strains in regard to biofilm formation and sanitizer tolerance (50, 54, 117–121). Taylor and Stasiewicz found no difference between these two classes of isolates when planktonic growth was examined under salt, pH, and QAC sanitizer stress, as well as under different energy sources (120). One study done on isolates from a wide range of food processing environments found that biofilm formation was unpredictably variable, and concluded that there was no connection between an enhanced ability to form biofilm and persistence in food processing environments (122). Another study which examined isolates from gorgonzola cheese processing plants also found that there was no link between persistence and phenotypic biofilm forming ability or sanitizer tolerance (117). Additionally, a different study found that there was no link between an isolate being a strong biofilm former and an increased tolerance to sanitizer (54). Assisi et al. examined the genomes of persistent and transient *L. monocytogenes* from retail delis, as well as the global biofilm and planktonic transcriptomes (50). They found no genomic content to support or explain the persistent status of isolates, and did not observe the expected changes in genetic expression (50). Overall, evidence to whether persistence is linked to these bacterial abilities is inconclusive, and more research is needed.

Biofilm and Sanitizers Together

Several studies have observed that to sanitize biofilms properly, an increased amount of sanitizer is needed compared to planktonic cells (84, 123, 124). In a comparison study of

20 sanitizers, the only sanitizer groups that were able to effectively sanitize a biofilm with a 5-log reduction in cell counts were acidified sodium chlorite, chlorine dioxide, and peroxyacetic acid (123). Chlorine and QAC were among the worst performers against biofilm, but all except one biguanide-based sanitizer were able to reduce planktonic cells by 5-log at or below manufacturer recommendation (123). Recent work by Andrade et al. also showed QAC had minimal effect on 5-day *L. monocytogenes* biofilm (125), but Belessi et al. determined that QAC was the most effective sanitizer on acid-adapted biofilms up to 12 days old (126). On the other hand, Cabeça et al. determined sodium hypochlorite was most effective on 5-day *L. monocytogenes* biofilms (110), which is in agreement with work done by Rodrigues et al. which observed that sodium hypochlorite was the most effective sanitizer and needed the lowest concentration to eradicate 24-hour *L. monocytogenes* biofilms (127). These studies are all done under different conditions, with different sanitizers as treatment, and use either reference *L. monocytogenes* strains or a set of isolates from the specific conditions being studied. This variability has the potential to change the results significantly between studies, and comparison of conclusions should be done with care.

Pan et al. studied biofilm formation and sanitizer tolerance of five *L. monocytogenes* strains from food on both Teflon and stainless-steel surfaces in a complex simulated food processing model (52). It was observed that the biofilms adapted a tolerance to the repeated peroxide sanitizer treatment over the course of three weeks (52). They found that reduction in cell counts was greater on the stainless steel-grown biofilms regardless of sanitizer treatment, and exposure of biofilms to peroxide sanitizer provided cross-protection for QAC and chlorine sanitizer (52). However, when they applied

sanitizer to cells removed from treated biofilms, they saw reductions that suggest tolerance to sanitizers was due to a feature of the biofilms themselves and not intrinsic properties of the cells (52). The biofilm model used in this circumstance most closely represents a food production environment and the associated stressors and provides strong evidence that sanitizer tolerance of biofilms is a result of the structure, rather than composition of cells (52).

Some studies have revealed additional interesting factors affecting biofilms tolerance to sanitizer. Lourenco et al. found that biofilms formed at 12°C were not as susceptible to sanitizers, revealing that cold stress may be a factor that plays into making biofilms more tolerant to eradication in food production environments (88). This is an important point of focus because in many processing facilities the room temperature is not what would be considered comfortable at around 22°C, but much cooler near refrigeration temperatures. Locations such as refrigerators, walk-in coolers, and other cold storage solutions are also important to consider, especially for a psychrotroph like *L. monocytogenes*. Another thing rarely considered in these works is the potential for sanitizer residues to remain behind and give certain bacteria an advantage. Ortiz et al. observed that in the absence of benzalkonium chloride (BAC), BAC-tolerant strains of *L. monocytogenes* were disadvantaged, and BAC-sensitive strains grew to higher levels. In the presence of sub-inhibitory concentrations of BAC, however, the resistant strains had the advantage (128). This finding is pertinent because not all sanitizers function the same, and there will be residues left behind by QACs.

Summary: Implications for Artisan Dairy and Knowledge Gaps

L. monocytogenes is a long-established threat to the safety of raw milk and artisan cheese overall and remains a problem today. Vermont is a hub of artisan cheese making and consumption, and this food is closely linked to the experience of being a Vermonter (29). This review finds that biofilm formation and sanitizer tolerance are likely mechanisms that will allow *L. monocytogenes* to persist in food processing environments, and possibly including farmstead artisan dairies. Though work has been done to identify if there is pathogen present in the raw milk being used for Vermont cheeses (18) or the environments they are produced in (26), there is still a knowledge gap about the qualities of these pathogens that may make them more or less dangerous to the consumers who are so enthusiastic about these products. Increased biofilm formation and sanitizer tolerance could potentially be linked to *L. monocytogenes* persistence in Vermont artisan dairies, resulting in increased risk to local consumers as well as increased risk for producers and viability of this business.

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Biofilm Sanitizer Tolerance of Vermont Dairy *Listeria monocytogenes*

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Abstract

Listeria monocytogenes is a foodborne pathogen found in biofilms on surfaces and equipment in the food processing environment. Sodium hypochlorite (SH) and quaternary ammonium compounds (QACs) are readily available and commonly used sanitizers. However, due to the structure and additional organic material produced in a biofilm, killing bacteria within biofilms may be a challenge for one or both of these sanitizers. The objective of this work was to determine if planktonic cells and immature and mature biofilms from *L. monocytogenes* isolated from Vermont artisan dairy environments could be effectively eliminated by SH and QACs. To determine sanitizer minimum inhibitory concentration (MIC) for planktonic cells, cultures were incubated statically in 1x or 1/20x Brain Heart Infusion broth (BHI) in polystyrene microtiter plates for 24 hours at room temperature (22°C) with serial dilutions of sanitizer. Sanitizer efficacy on biofilms was determined by growing isolates on one cm stainless steel coupons in 1x or 1/20x BHI to simulate nutrient rich and poor conditions on a surface commonly seen in food processing environments. Coupons were incubated statically for 1, 3, or 10 days. Media was replaced every 48 hours to prevent nutrient depletion. After incubation, coupons were rinsed 3 times with phosphate buffered saline and placed into 0, 50, 100, or 200ppm SH or QAC for the manufacturer recommended contact time. Sanitizer was neutralized and adherent cells were removed by vortexing with beads. Cell suspensions were diluted and plated, then counted via spot plating on BHI agar. Significant differences for biofilm survival were assessed using Analysis of Variance in R (v.4.0.4). MIC for isolates grown in nutrient poor (1/20X BHI) conditions were lower than nutrient rich conditions (1X BHI) for both sanitizers. All isolates' biofilms reached ~6-8 log10 CFU/coupon on stainless steel. Reductions from different QAC concentrations differed (p_{adj} <0.05) in 1/20x BHI but were not significantly different in 1x BHI. In both biofilm growth conditions SH was more effective at 200 ppm than 50 ppm (p_{adj} < 0.05). Biofilms from both persistent and transient *L. monocytogenes* environmental isolates from Vermont dairies are resistant to working concentrations of QAC sanitizer, but sodium hypochlorite bleach reduces *L. monocytogenes* biofilm on stainless steel.

Importance

This study examines the potential for innate persistence characteristics in *Listeria monocytogenes* from an infrequently studied source, Vermont artisan dairies. It aims to determine if a set of environmental isolates have increased tolerance to quaternary ammonium compound and sodium hypochlorite, as well as increased biofilm forming ability that would enable persistence in food production environments.

Keywords: *Listeria monocytogenes,* artisan dairy, biofilm, sanitizer tolerance

Introduction

Listeria monocytogenes is a foodborne pathogen which is specifically problematic in dairy foods. Most dairy foods are low in salt, have a high water activity, and contain nutrients that *L. monocytogenes* can thrive on (129). Additionally, *L. monocytogenes* can grow at temperatures as low as 4°C and will multiply in foods such as milk or cheese at refrigerator temperatures that inhibit the growth of other pathogenic or spoilage organisms (3). This bacterium is found in the natural environment in soil or water, asymptomatically in dairy animals, and in biofilms inside of food processing plants (65, 130). Therefore, there are many possible routes of contamination with *L. monocytogenes* during dairy production, especially in farmstead or artisan cheesemaking operations where the dairy and farm environment is incredibly close to food production facilities (131). *L. monocytogenes* can persist in these environments, or be repeatedly introduced over time from contaminated ingredients, employee movement, or the environment. *Listeria* control in all dairy operations is a constant campaign of cleaning and sanitizing to ensure that cross contamination is limited, and product does not contain any pathogen.

Unfortunately, *L. monocytogenes* can form biofilms, which are dynamic multispecies communities of bacteria that are protected by an extracellular polysaccharide matrix (63). These biofilms are a safe harbor for pathogens, providing an

increased level of safety from detergents and sanitizers as well as protection from changing environmental conditions (63). They form in hard-to-reach and rarely cleaned locations such as in floor drains or in crevices of equipment not designed to be disassembled (70). Biofilms are linked to persistence in several types of food processing environments and can shed onto other surfaces or into foods (64).

Cleaning and sanitizing appropriately is the best way to eliminate biological hazards and prevent biofilm formation. However, certain *L. monocytogenes* isolates have previously demonstrated the ability to tolerate sanitizers used in food production (99). Resistance is when a bacterium is able to survive in a concentration of an anti-bacterial substance, like an antibiotic, that would normally kill it (104), but tolerance is a better representation of the phenomenon of pathogens requiring a higher than usual concentration of sanitizer for disinfection (105). Sanitizers at recommended concentrations should result in a 5-log reduction of bacteria, and are considered effective at this level (132). Quaternary ammonium compounds (QACs) are sanitizers commonly used on food contact and non-food contact surfaces because of their broad-spectrum killing capacity and ability to remain stable at a wide range of pH and over extended periods of time (99). Sodium hypochlorite (SH) is another common sanitizer that is used in food processing; it is easily accessed and a powerful oxidizer, but concentration must be monitored frequently as it degrades after exposure to light, heat, and organic material (103).

L. monocytogenes is a common problem and as such, it is commonly studied. However, there is a lack of information about it in the context of smaller-scale dairy operations. D'Amico and Donnelly conducted environmental surveys of eight small

Vermont artisan dairy operations in 2008 and found that *L. monocytogenes* prevalence was 2.1% among 236 environmental samples (26). In 2006, these facilities were positive for the same *L. monocytogenes* ribotypes (26). While they identified low contamination levels, they did not examine phenotypic or genotypic factors associated with persistence (26).

Our objective was to test *L. monocytogenes* isolates from small cheesemaking facilities to determine the level at which planktonic cultures at nutrient rich and nutrient poor conditions will tolerate QAC and SH, to observe their capacity to form biofilm in these conditions and the ability of resulting biofilms to tolerate challenge with full and diminished strength sanitizer.

Materials and Methods

Long-term Storage and Recovery of Selected Isolates

Isolates used in this work were obtained from the Donnelly culture collection and were stored in 25% glycerol and Trypticase Soy Broth (TSB, BD, Franklin Lakes, NJ) in -80°C for long term storage. We selected 31 isolates to represent a variety of ribotypes, lineages, and source locations. They consist of *L. monocytogenes* from lineages I and II, and 15 of the 31 isolates have DUP-ID 1042 which was considered to be the persistent ribotype because it was the most prominent and most frequently re-isolated across the original study (26). Isolates were recovered from frozen stocks by thawing at room temperature and streaking 5 uL onto Brain Heart Infusion (BHI, Difco, Life Technologies, Detroit, MI) agar and incubating at 37°C overnight. One isolated colony was used to inoculate BHI broth and incubated at 37°C for 18 hours. Overnight broth

culture was added at a 1:1 ratio of culture to 50% glycerol in cryovials for storage at -80°C as working stock for the duration of these experiments.

Planktonic *Listeria monocytogenes* MIC Determination

Minimum Inhibitory Concentration (MIC) for sanitizers was determined as follows, based on methods from Wang et al. (121)

Preparation of Culture:

Isolates were recovered from the working stock solutions by streaking 5 uL onto BHI agar and incubating at 37°C overnight. After 24 hours, 8 to 10 isolated colonies were selected with a sterile swab and suspended in 5mL of BHI broth. Optical density (OD_{600}) of the culture was read with Epoch microplate spectrophotometer (BioTek, Winooski, VT) and adjusted to a range of OD_{600} 0.124-0.140. Adjusted culture was then diluted 1:100 into 1mL of 2x concentration BHI or 1/10x concentration BHI; media was later further diluted by addition to sanitizer, resulting in full strength or $1/20^{th}$ strength BHI to approximate nutrient rich and nutrient poor conditions.

Preparation of Sanitizer Dilutions:

Solutions of sodium hypochlorite (SH, Clorox, Oakland, CA) and J-512 quaternary ammonium sanitizer (QAC, Diversey, Fort Mill, SC) were prepared to achieve concentrations of 6400ppm and 200ppm, respectively. Aliquots of 200uL of sanitizer were added to 7 wells of the first column of a 96-well polystyrene microtiter plate (9017 Corning, Corning, NY). In the next 5 columns, 100uL of sterile deionized water (SDW)

was added to 7 wells. Sanitizer was serially diluted 1:2 in wells containing SDW, and then 50uL of each sanitizer concentration was transferred to the remaining 6 empty columns, creating a duplicate of the first half of the plate. 50uL of each sanitizer concentration was transferred to the.

Plate Inoculation and Incubation:

Each 96-well plate contained enough wells to test three isolates in duplicate at nutrient rich (1x BHI) and nutrient poor (1/20x BHI) conditions. 50uL of adjusted culture in 2x BHI was added to the first set of 2 rows of sanitizer concentrations, and 50uL of adjusted culture in 1/10x BHI was added to the second half of rows A-F for each isolate in duplicate. This resulted in 100uL each of 3200, 1600, 800, 400, 200, and 100ppm SH, and 100, 50, 25, 12.5, 6.25, and 3.125ppm QAC, and 1x and 1/20x BHI. For negative controls, 50uL of sterile media was added to the 7^{th} well (row G) of the plate; 2x BHI was added for the first set of sanitizer concentrations (column 1-6), and 1/10x BHI was added for the second set (column 7-12). For positive controls, 50uL of 2x and 1/10x sterile media was added to the bottom row of the plate and inoculated with 50uL of adjusted culture for each isolate at both nutrient conditions in duplicate. Plates were read at hour 0 immediately following inoculation to determine baseline OD_{600}, and again to measure growth following static incubation for 24 hours at room temperature (22°C). At 24-hour reading, test wells were compared to negative control OD_{600} measurement, and MIC was determined to be the concentration of sanitizer where no growth occurred. Growth was defined as having a greater value than negative control of respective sanitizer concentration.

Standard Crystal Violet Biofilm Assay

Preparation of Culture:

Isolates were streaked onto BHI agar plates and incubated at 37°C for 24 hours. A single isolated colony was aseptically selected to inoculate BHI broth and incubated at 37°C with shaking (200rpm) for 18-24 hours. Overnight cultures were diluted in BHI broth and OD_{600} adjusted between 0.05-0.10 using Epoch spectrophotometer.

Microplate Set-up:

Three 96-well microplates were prepared as follows. Adjusted culture was added to wells in triplicate for each environmental isolate. Sterile BHI broth was added to three wells as a negative control. Microplate was incubated at room temperature (22°C) for 1, 3, or 5 days.

Microplate Washing, Staining, and Visualization:

One microplate was washed and stained for visualization on day 1, 3, and 5 of incubation. Cells and spent media were removed from wells and discarded. Wells were rinsed with sterile deionized water 3 to 4 times to remove cells not contained in biofilm. After washing, 150uL of 0.1% aqueous crystal violet was added to each well and incubated for 30 minutes to stain. After 30 minutes of staining, crystal violet was removed from wells and microplate was rinsed with water until water ran clear. Plates were inverted for storage to dry. After drying, 100uL of 95% ethanol was added to each well to elute

crystal violet from biomass. Ethanol was transferred to corresponding well in a new sterile 96-well microplate. Optical density was measured using Epoch microplate spectrophotometer to determine crystal violet retention as an approximation of biomass generated.

Evaluating Sanitizer Efficacy on *Listeria monocytogenes* Biofilm

Preparation of Culture:

Frozen stocks of *Listeria monocytogenes* isolates were streaked onto BHI agar and incubated at 37°C for 24 hours. An isolated typical colony was aseptically selected to inoculate BHI broth. Broth was incubated at 37°C with shaking (200rpm) for 18-24 hours. Liquid culture was added to either full strength media (1x BHI) or reduced strength media (1/20x BHI) and then OD_{600} adjusted between 0.06-0.10, read on Epoch microplate spectrophotometer.

Preparation of Stainless-Steel Coupons and Glass Beads:

Stainless-steel coupons (AISI 304, 1.2mm thick, 10mm diameter, machined by UVM Instrumentation and Model Facility) and glass beads (solid soda lime #3000, 2mm diameter, Andwin Scientific, Simi Valley, CA) were soaked in 100% acetone for 30 minutes to remove residues from manufacture, then scrubbed with constant manual agitation in liquid dish detergent (Dawn, Procter & Gamble, Cincinnati, OH) for 5 minutes and rinsed 5 times until water ran clear with deionized water to remove soap residue, followed by an ethanol rinse before autoclaving at 121°C for 15 minutes to sterilize.

Formation of Biofilm:

Sterile coupons were aseptically transferred with sterile forceps to empty petri dishes (Fisher Scientific, Pittsburgh, PA) prior to inoculation. Petri dishes containing 4 coupons each were inoculated with 10mL adjusted liquid culture in 1x or 1/20x BHI broth to simulate nutrient rich and nutrient poor conditions. Coupons were incubated statically at room temperature (22°C) for 1 day and 3 days to represent immature biofilms, or 10 days to represent mature biofilms. To prevent nutrient depletion, spent media was aseptically removed and replaced every 48 hours on days 2, 4, 6, and 8 of incubation. Experiments for each isolate were repeated three times on separate days. All 31 environmental isolates were tested at mature conditions, and a representative subset of 6 isolates were tested at immature conditions.

Sanitizer Application to Biofilm and Enumeration:

After incubation, media was removed from the petri dish and coupons were rinsed 3 times with 20mL aliquots of phosphate buffered saline (PBS) to remove unadhered cells from the surface. Rinsed coupons were transferred with sterile forceps to test tubes containing 1mL of 0, 50, 100, or 200ppm sanitizer. Sanitizers used were quaternary ammonium compound J-512 (QAC) and sodium hypochlorite (SH). After the manufacturer recommended contact time of 60 seconds for QAC and 2 minutes for SH, 9mL of Dey-Engley broth (D/E, Difco, Life Technologies, Detroit, MI) was added to neutralize the sanitizer. Sterile glass beads (1 +/- .1g) were added, and coupons were vortexed for 2 minutes to remove adherent cells into suspension. Samples were serially

diluted in buffered peptone water (BPW, Difco, Life Technologies, Detroit, MI), and duplicate plated onto BHI agar using a 20uL spot plate method (133). Plates were incubated for 24 hours at 37°C prior to enumeration.

Statistical Analysis:

Significant differences for cell survival of sanitizer treatment and decrease compared to control were assessed using Analysis of Variance followed by Tukey's honest significance test in R (v. 4.0.4) (134). The models created used log transformed count data as a function of isolate and sanitizer concentration for each day and nutrient condition, i.e. Count~isolate+sanitizer concentration+isolate*sanitizer concentration.

Results

Sanitizer MIC

At room temperature (22°C), average minimum inhibitory concentrations of QAC in nutrient rich conditions ranged from 3.75 to 14.58ppm. In nutrient poor conditions, average MIC ranged from 1.88 to 6.25ppm. These ranges are far below the manufacturer recommended concentration for this sanitizer, which is 200ppm for food contact surfaces and 400ppm for non-food contact surfaces.

SH minimum inhibitory concentrations for isolates grown in nutrient poor (1/20X BHI) conditions ranged from 25 to 400ppm, and in nutrient rich conditions (1X BHI) from 25 to 1600ppm.The higher ends of these MIC ranges surpass the manufacturer recommended concentration of SH, which is 200ppm for food contact surfaces.

There were no significant differences among isolates for SH tolerance, and minimal differences for QAC tolerance. Isolate 21 had a greater tolerance to QAC than isolate 7, and both isolate 20 and 23 showed greater tolerance compared to isolate 4 ($p<0.05$). For both sanitizers, there was a greater tolerance exhibited by all isolates grown under the nutrient rich condition compared to the nutrient poor condition. Overall, the isolates did not tolerate QAC well, and MICs were much lower than recommended working concentration, but many isolates had higher SH MICs than the concentration recommended for treating food contact surfaces.

Biofilm Growth on Polystyrene and Stainless Steel

When isolates were grown for the microplate assay in the nutrient rich condition, we observed significantly greater biomass on day 5 compared to both day 3 and day 1

($p<0.05$). In the nutrient poor condition, there was an increase in optical density from day 1 to day 3 but not from day 3 to day 5, so cell populations leveled off at these two later time point. Isolate 29 had significantly greater growth than 28 of 30 other isolates in the nutrient rich condition. Under the nutrient poor condition, isolate 10 exhibited significantly greater growth compared to all 30 other isolates. No other between-isolate differences were observed, which suggests that neither of these isolates is particularly remarkable on the whole.

Though attachment on polystyrene varied slightly, all isolates reached ~6-8 log10 CFU/coupon on stainless steel when enumerated after 10 days with no significant differences between isolates. Growth at both nutrient rich and poor conditions reached this level.

Sanitizer Efficacy on Biofilm

Generally, biofilms incubated in nutrient poor media were less tolerant to both sanitizers, and biofilms incubated in nutrient rich media were more resistant to both sanitizers. QAC treatment on biofilms from nutrient rich conditions was equally effective for days 1, 3, and 10. Under nutrient poor growth conditions, QAC treatment was more effective on day 10 compared to days 1 and 3 ($p<0.05$). SH was more effective at earlier time points (days 1,3) than on day 10 at both conditions ($p<0.05$).

Three sanitizer concentrations (50, 100, and 200ppm) of QAC and SH were applied to represent the manufacturer recommended working strength for food contact surfaces (200ppm) and reduced strength sanitizer as might be found when working stocks are improperly formulated or remain as residue in a food production environment. SH

was more effective than QAC for all timepoints, but neither was consistently effective at decreasing cell counts to levels needed to consider the surface sanitized; the ideal reduction would be a 5-log decrease (132). These sanitizers alone are not able to eliminate *L. monocytogenes* biofilm on stainless steel.

Sanitizer Efficacy on 1-day Biofilms

On the least developed biofilms QAC was not effective at reducing cell numbers of *L. monocytogenes* biofilms, with the greatest reduction being ~1-log decrease. At both 1-day nutrient conditions, all QAC concentrations performed similarly. Biofilms grown at the nutrient rich condition exhibited no difference between isolates when treated with QAC after 1 day. At the nutrient poor condition, isolate 7 was more susceptible to treatment with QAC compared to 4/5 other isolates ($p<0.05$).

SH was better than QAC at killing 1 day old *L. monocytogenes* biofilms, reaching a 6-log decrease; nutrient rich and nutrient poor biofilms were both similarly affected by SH. Full strength 200ppm sanitizer worked best on 1 day old biofilms at both nutrient conditions ($p<0.05$), while 100ppm reduced-strength sanitizer was more effective than 50ppm when applied to biofilms from the nutrient poor media ($p<0.05$). Isolate 10 incubated at nutrient poor conditions was consistently reduced to below the detectable limit when treated with 200ppm SH.

Sanitizer Efficacy on 3-day Biofilms

QAC was ineffective at treating 3 day old *L. monocytogenes* biofilm. Isolate 7 was the most sensitive to treatment, but reductions did not reach 2-log with any sanitizer

concentration used, showing that even this most sensitive isolate was barely disrupted. Biofilms incubated for 3 days in nutrient poor conditions were more affected by QAC than nutrient rich biofilms, but overall, both nutrient conditions resulted in hardy biofilms that stood up to all QAC concentrations tested.

SH treatment was effective on all 3 day *L. monocytogenes* biofilms whether they were grown in nutrient rich or poor conditions. All isolates tested showed a maximum of 4-log and 5-log reduction at nutrient rich and poor conditions, respectively. As SH concentration increased, effectiveness improved, with 200ppm resulting in significantly greater reductions than 50ppm ($p < 0.05$).

Sanitizer Efficacy on 10-day Biofilm

L. monocytogenes isolates grown for 10 days in nutrient rich conditions were overall tolerant to QAC application. Isolates in these conditions were all equally sensitive to treatment, and decreases after QAC application did not surpass 2-log. However, when grown under nutrient poor conditions there were some differences in isolate response to QAC treatment. Isolate 4 was observed to be more sensitive than 10 other isolates, isolate 7 was more sensitive than 4 other isolates, and isolate 6 was more sensitive than 2 other isolates when treated with QAC. Though decreases were greater with greater concentrations of QAC ($p<0.05$), they did not consistently reach the sanitizing standard of a 5-log reduction. Therefore, even the highest concentration tested (200ppm) applied as recommended by the manufacturer for food contact surfaces, was not adequate to kill *L. monocytogenes* biofilms.

SH was better than QAC at reducing levels of viable *L. monocytogenes* in biofilms grown in nutrient rich and nutrient poor conditions. Decreases on nutrient rich biofilms reached as high as 4-log, and, on nutrient poor biofilms, as high as 6-log. SH efficacy increased with increased concentration, and in both growth conditions the recommended working concentration of 200ppm resulted in the greatest log reductions ($p<0.05$). Overall isolates responded similarly to treatment with SH, but isolate 3 was less tolerant to sanitizer compared to one other isolate at nutrient rich conditions, and less tolerant than three other isolates in nutrient poor conditions.

Discussion

Overall, we have found that *L. monocytogenes* biofilms from VT dairy environmental isolates are robust and resist elimination by sanitizers alone. The quaternary ammonium compound-based sanitizer that we utilized for these experiments performed particularly poorly on biofilms at all three time points and under all nutrient conditions (**Figure 1 and 2**). In some circumstances biofilm cell counts seemed to increase from the control condition after sanitizer application, as is reflected in **Figures 1, 2** and **Table 2**. This is merely an artifact of the minimal effect the QAC had on the biofilm; the sanitizer could not kill enough cells to account for variation in the original biofilm growth between coupons.

However, this QAC did perform well in MIC assays targeting planktonic cultures (**Table 1**). The fact that the sanitizer performs better on planktonic cells is also supported by others; Cruz and Fletcher previously observed that biofilms resist sanitizer more than planktonic cells (123). Pan et al. observed that cells from sanitizer stress-adapted biofilms did not have any acquired increase in tolerance to sanitizer; this suggests the biofilm itself, rather than intrinsic characteristics of the cells within it, is the primary mechanism of sanitizer tolerance (52). This aligns with the results we saw with our QAC application. The structure of QAC interacts with the cell membrane to disrupt and kill cells, and the structure of the sanitizer is preserved for additional membrane disruption and killing. If encountering a polysaccharide matrix of a biofilm, the action of these particles will be hindered even if they do not degrade. In assays we performed where there was little additional organic material surrounding cells in their planktonic form, QAC performed well (**Table 1**). But, where the cells were protected by a matrix in all of

the biofilm formation conditions, the killing effect of the QAC was decreased (**Figure 1 and 2**). In certain circumstances, researchers have observed that QACs can even make biofilms grow better. At subminimal levels QAC can enhance the biofilm formation of resistant *L. monocytogenes* strains (135). *L. monocytogenes* isolates have also demonstrated enhanced biofilm formation at 4°C when in the presence of QAC (136).

Where QAC performed very poorly eliminating biofilm, SH had a much greater effect. In all biofilm nutrient conditions, time points, and for all isolates, SH caused greater reductions in cell counts compared to QAC(**Figure 3 and 4**). Previously, SH has been shown as the most effective sanitizer compared to other household cleaners, and in our work it was also more effective compared to QAC (111). SH is a strong oxidizer and will react with organic material. It is this mechanism in interaction with cellular components that causes damage and destruction of cells. The mechanism of sanitizer is indiscriminate, so any organic material that SH encounters will be degraded, and as a result the SH will be inactivated. This mechanism may be advantageous when a biofilm is encountered, because the matrix could be disrupted, and cells inside would no longer be protected from the external environment. Many researchers have used microscopy to observe the basic structure of *L. monocytogenes* biofilms (82, 91, 137); further work on these isolates could be focused on SEM observation before and after sanitizer application to determine the extent of any structural disruption caused by SH.

SH was not as effective in planktonic MIC assays as QAC (**Table 1**). The increased strength of sanitizer necessary to inhibit growth could be due to the different characteristics of SH, namely that it will degrade with exposure to light and exposure to organic material. This reduced effectiveness seen in the MIC microtiter plate assay may

be due to the presence of rich lab media, which would inactivate sanitizer upon reaction, and leave none behind to continue inhibiting cells as they grew overnight. The format of such an assay does not allow us to know what type of live cell reductions are happening at time of sanitizer addition, and cells remaining could easily multiply and continue to inactivate SH as they grow. We observed increased tolerance to higher concentrations of SH in this MIC assay. This goes against what we would expect; if the sanitizer were affecting the more complex biofilm system, it should affect the planktonic cells similarly or better. Further investigation of this question would be necessary to determine if this is truly a tolerance of these *L. monocytogenes* isolates to SH or if it is an artifact of the mechanism's cross-reactivity with other organic material and subsequent inactivation.

In this work, we examined the effect of relatively high and low nutrient conditions on the survival of *L. monocytogenes* biofilm. For the crystal violet (CV) plate assay, the differences in observations between nutrient rich and nutrient poor conditions could be related to nutrient depletion or buildup of waste in the nutrient poor condition. In the nutrient rich condition, there was a higher availability of nutrients at the start, which would sustain the inoculated population for presumably a longer period of time and result in the observed continuous increase in biomass. In the nutrient poor condition where there were less available nutrients at the outset, the cells may have experienced growth and then die off, thus exhibiting lower biomass in later time points at this growth condition.

For the stainless-steel biofilm assays, *L. monocytogenes* grew well in both nutrient conditions, and control growth levels were only slightly reduced in the nutrient poor condition (**Table 2**). This is likely due to continuous replenishment of media every 48

hours; with a constant supply of nutrients even at lower concentrations the population of *L. monocytogenes* in this single-species biofilm grew well. This observation is supported by the literature, as others have seen that *L. monocytogenes* is capable of forming biofilm under both nutrient rich and poor conditions, but will form greater amounts under nutrient rich conditions (138–140). However, it has been shown that with different growth models a biofilm formation outcome can be affected; for example, the system has a continuous addition of nutrients *L. monocytogenes* biofilm may grow differently (141).

The model of biofilm growth used here has limits. It is a monospecies model, which controls for the interactions between other bacteria. But, biofilms are realistically never composed of only a single species of bacteria and are rather dynamic and complex communities of many organisms (63). This model incorporates static incubation and does not account for any liquid movement that may take place in a location where biofilms grow in food production environments such as pipes and drains. In food production environments there is also a repeated cycle of nutrient supply, sanitizer application, and drying that we did not consider in our model. The model used by Pan et al. mimicked this cycle more closely; a better approximation of realistic conditions facilitates drawing rational conclusions (52).

The interaction between biofilm maturity and sanitizer effect was not particularly telling for QAC treatment, which was largely ineffective on all days (**Figure 1 and 2**), but SH was more effective at earlier biofilm time points (**Figure 3 and 4**). Our work reflects what has been seen before; Fagerlund et al. observed that 7-day biofilms were more resistant to cleaning than 4-day biofilms, which is a similar observation to that of Papainnou et al., who observed 7-day biofilms being more resistant than 3-day biofilms

(91, 142). Another study noted that there was reduced sanitizer efficacy on more mature biofilms grown on polystyrene (143). Additionally, a study examining *L. monocytogenes* biofilms grown on high density polyethylene used in cutting boards showed that sanitizer had greater efficacy on biofilms that had been incubated for shorter amounts of time (144).

As a whole, this work reinforces that sanitizer cannot be used alone to eradicate biofilm at concentrations that manufacturers recommend for normal use. These chemical sanitizers are meant to kill cells, not disrupt significant organic material. Biofilms are complex structures, and the matrix serves as a protective barrier from the outside world. Sanitizers work best on a cleaned surface, one which has been treated with chemical or mechanical means of disruption to organic compounds such as lipids, proteins, or the EPS matrix of a biofilm. In practice, food producers both large and small must focus on the entire cleaning and sanitizing process, using these combined efforts to disrupt then kill biofilm, because sanitizer is not effective alone.

Acknowledgements

A.E. conceived of the idea for the experiments. Crystal Violet assays were done by L.C., QAC MICs were determined by A.G., SH MICs and Evaluation of Sanitizer Efficacy on *L. monocytogenes* Biofilms along with data compilation and analysis were completed by E.F..

Tables

Table 1: *L. monocytogenes* environmental isolate planktonic minimum inhibitory concentrations of sanitizer. All *L. monocytogenes* isolates from Vermont artisan dairy environments. Isolates with asterisk were used in immature biofilm (1-day and 3-day) assays.

Isolate	Ribogroup	DUP ID[a]	Source	Lineage	QAC MIC 1x,1/20x[b]	SH MIC 1x,1/20x[c]
FML-F1-0001*	ECORI 210-511-S-7	18645	Floor (entrance)	-	5.63, 4.38	666.67, 225
FML-F1-0002	ECORI 210-506-S-5	1042	Floor (under vat)	-	6.25, 3.13	800, 225
FML-F1-0003	ECORI 210-506-S-5	1042	Drain (aging room 2)	-	6.25, 6.25	800, 225
FML-F1-0004	ECORI 210-506-S-5	1042	Drain (aging room 1)	-	3.75, 1.88	1066.67, 162.5
FML-F1-0005	ECORI 210-506-S-6	1006	Shoes	-	6.25, 6.25	1066.67, 150
FML-F1-0006	ECORI 210-506-S-5	1042	Floor squeegee	-	6.25, 3.13	1066.67, 175
FML-F1-0007*	ECORI 210-506-S-5	1042	Drain (aging)	I	5, 1.88	733.33, 83.33
FML-F1-0008	ECORI 210-506-S-1	19157	Drain (aging)	II	8.33, 4.17	800, 116.67
FML-F1-0009*	ECORI 210-506-S-1	19171	Floor squeegee	I	6.25, 3.13	800, 116.67
FML-F1-0010*	ECORI 210-506-S-5	1042	Cut and wrap table	I	6.25, 5.21	800, 133.33
FML-F1-0011	ECORI 210-506-S-1	1030	Drain (vat)	II	6.25, 6.25	800, 166.67
FML-F1-0012	ECORI 210-506-S-1	1039	Drain (vat)	II	6.25, 6.25	800, 166.67
FML-F1-0013	ECORI 210-506-S-5	1042	Pooled water	I	6.25, 5.21	800, 83.33
FML-F1-0014	ECORI 210-506-S-5	1042	Water hose	I	8.33, 5.21	800, 116.67
FML-F1-0015	ECORI 210-512-S-4	19171	Drain (vat)	-	6.25, 5.21	733.33, 116.67
FML-F1-0016	ECORI 210-506-S-1	19157	Pooled water	-	7.19, 6.25	1000, 180
FML-F1-0017	ECORI 210-506-S-1	19157	Pooled water	II	6.25, 6.25	800, 133.33
FML-F1-0018	ECORI 210-508-S-8	1039	Floor (Entrance)	-	11.46, 5.21	800, 83.33
FML-F1-0019*	ECORI 210-506-S-5	1042	Bucket (fill hoops)	I	12.5, 5.21	810, 141.67
FML-F1-0020	ECORI 210-513-S-5	10144	Floor squeegee	-	8.33, 6.25	800, 100
FML-F1-0021	ECORI 210-506-S-5	1042	Hallway floor	I	14.58, 6.25	666.67, 100

FML-F1-0022	ECORI 210-506-S-1	1039	Drain (new)	-	4.69, 4.69	800, 100
FML-F1-0023	ECORI 210-511-S-7	18645	Drain (vat)	II	14.58, 5.21	800, 100
FML-F1-0024	ECORI 210-506-S-5	1042	Drain (aging)	I	6.25, 3.13	800, 100
FML-F1-0025	ECORI 210-506-S-5	1042	Drain (aging #2)	I	8.33, 3.13	933.33, 200
FML-F1-0026	ECORI 210-506-S-1	19171	Floor squeegee	I	6.25, 4.17	933.33, 233.33
FML-F1-0027	ECORI 210-506-S-5	1042	Cut and wrap table	I	6.25, 3.13	1066.67, 233.33
FML-F1-0028	ECORI 210-511-S-7	18645	Drain (vat)	II	6.25, 4.17	933.33, 133.33
FML-F1-0029*	ECORI 210-506-S-5	1042	Hallway floor	I	5.63, 2.08	933.33, 166.67
FML-F1-0030	ECORI 210-506-S-5	1042	Cut and wrap table	I	6.25, 4.17	933.33, 200
FML-F1-0031	ECORI 210-506-S-1	19157	Pooled water	-	-,-	1333.33, 225

[a] DUP-ID, DuPont Identification Library codes, based on *Eco*RI ribotyping
[b] QAC, quaternary ammonium compound sanitizer; MIC, minimum inhibitory concentration required to prevent growth
[c] SH, sodium hypochlorite sanitizer; MIC, minimum inhibitory concentration required to prevent growth

Table 2: *L. monocytogenes* environmental isolate mature 10 day biofilm growth and response to sanitizer application.

BHI Concentration	Sanitizer Concentration	Isolate	Control (0ppm) Log10 CFU/coupon	Log10 CFU/coupon	Log10 Decrease from Control
Quaternary Ammonium Compound					
1/20x	50ppm	Average	6.32	5.80±0.49	0.52
		1	6.76	6.24± 1.44	0.52
		2	6.55	6± 0.34	0.55
		3	7.07	6.24± 0.53	0.83
		4	6.32	5.35± 1.24	0.97
		5	7.52	6.38± 0.31	1.14
		6	7.21	6.34± 0.16	0.87
		7	6.43	5.29± 0.65	1.13
		8	6.87	6.62± 0.46	0.25
		9	5.93	6.16± 0.74	-0.22
		10	5.87	5.73± 0.34	0.14
		11	5.56	5.36± 0.38	0.21
		12	6.45	5.9± 1.35	0.55
		13	6.67	5.95± 1.82	0.73
		14	6.65	6.05± 1.77	0.6
		15	6.3	5.69± 1.47	0.61
		16	6.56	6.36± 1.03	0.2
		17	5.79	5.01± 0.42	0.79
		18	6.12	5.83± 1.51	0.29
		19	7.06	6.59± 1.67	0.47
		20	5.85	6.12± 0.07	-0.27
		21	6.27	5.61± 0.35	0.66
		22	6.18	5.83± 0.52	0.35
		23	6.2	5.8± 0.55	0.4
		24	6.51	6.41± 0.63	0.1
		25	6.19	5.7± 0.43	0.49
		26	5.35	4.8± 0.27	0.54
		27	5.87	5.33± 0.47	0.54
		28	6.04	5.22± 0.18	0.82
		29	6.5	5.7± 0.27	0.79
		30	5.59	5.2± 0.57	0.4

BHI Concentration	Sanitizer Concentration	Isolate	Control (0ppm) Log10 CFU/coupon	Log10 CFU/coupon	Log10 Decrease from Control
Quaternary Ammonium Compound					
1/20x	50ppm	31	5.83	5.12± 0.53	0.71
	100ppm	Average	6.32	5.56±0.62	0.76
		1	6.76	6.05± 1.87	0.72
		2	6.55	5.51± 0.66	1.03
		3	7.07	5.72± 0.06	1.35
		4	6.32	4.65± 0.34	1.67
		5	7.52	6.58± 0.31	0.94
		6	7.21	5.85± 0.22	1.36
		7	6.43	5.16± 0.76	1.27
		8	6.87	6.55± 0.36	0.31
		9	5.93	5.87± 0.7	0.06
		10	5.87	5.39± 0.52	0.48
		11	5.56	4.28± 1.37	1.29
		12	6.45	6.13± 1.13	0.32
		13	6.67	5.91± 1.65	0.77
		14	6.65	5.86± 1.87	0.79
		15	6.3	5.86± 1.97	0.43
		16	6.56	5.57± 0.87	1
		17	5.79	4.73± 0.25	1.06
		18	6.12	5.31± 1.75	0.81
		19	7.06	6.73± 1.11	0.33
		20	5.85	5.9± 0.89	-0.06
		21	6.27	6± 0.2	0.27
		22	6.18	5.98± 0.82	0.21
		23	6.2	5.58± 0.51	0.62
		24	6.51	6.01± 1.21	0.51
		25	6.19	5.5± 0.7	0.69
		26	5.35	4.65± 0.09	0.7
		27	5.87	5.47± 0.61	0.4
		28	6.04	5.02± 0.55	1.02
		29	6.5	5.3± 0.41	1.2
		30	5.59	4.31± 0.5	1.29
		31	5.83	5.03± 0.5	0.8

BHI Concentration	Sanitizer Concentration	Isolate	Control (0ppm) Log10 CFU/coupon	Log10 CFU/coupon	Log10 Decrease from Control
Quaternary Ammonium Compound					
1/20x	200ppm	Average	6.32	5.04±0.75	1.28
		1	6.76	5.9± 1.55	0.86
		2	6.55	5.45± 1.13	1.09
		3	7.07	4.88± 0.16	2.2
		4	6.32	3.21± 2.38	3.12
		5	7.52	5.14± 0.96	2.38
		6	7.21	4.57± 0.37	2.65
		7	6.43	3.35± 3.03	3.08
		8	6.87	6.43± 0.51	0.43
		9	5.93	5.52± 0.98	0.41
		10	5.87	4.67± 0.03	1.21
		11	5.56	5.3± 0.32	0.26
		12	6.45	4.91± 2.07	1.54
		13	6.67	5.67± 2.16	1
		14	6.65	5.65± 1.62	1.01
		15	6.3	5.67± 1.57	0.63
		16	6.56	4.92± 0.62	1.64
		17	5.79	3.86± 0.33	1.93
		18	6.12	5.13± 2.29	0.99
		19	7.06	6.15± 1.52	0.91
		20	5.85	4.27± 1.1	1.58
		21	6.27	5.09± 0.69	1.18
		22	6.18	5.73± 0.77	0.46
		23	6.2	5.14± 0.3	1.06
		24	6.51	5.45± 0.89	1.06
		25	6.19	5.44± 0.66	0.75
		26	5.35	4.38± 0.49	0.97
		27	5.87	4.94± 0.8	0.93
		28	6.04	4.97± 0.74	1.07
		29	6.5	5.04± 0.6	1.45
		30	5.59	5.48± 0.56	0.11
		31	5.83	3.96± 0.8	1.88

BHI Concentration	Sanitizer Concentration	Isolate	Control (0ppm) Log10 CFU/coupon	Log10 CFU/coupon	Log10 Decrease from Control
Quaternary Ammonium Compound					
1x	50ppm	Average	7.45	7.23±0.54	0.22
		1	7.55	7.32± 1.55	0.23
		2	8.24	7.85± 0.26	0.39
		3	8.28	7.81± 0.22	0.47
		4	7.15	6.68± 0.88	0.47
		5	8.59	8.2± 0.02	0.39
		6	8.67	8.42± 0.22	0.25
		7	6.36	5.95± 0.89	0.41
		8	8.49	8.03± 0.29	0.46
		9	7.89	7.71± 0.48	0.18
		10	7.73	7.59± 0.29	0.14
		11	7.47	7.26± 0.83	0.21
		12	7.34	6.98± 0.66	0.37
		13	6.96	6.92± 0.45	0.04
		14	7.3	7.5± 0.22	-0.2
		15	7.23	6.51± 0.47	0.73
		16	7.39	7.06± 1.23	0.33
		17	7.16	6.77± 0.29	0.39
		18	6.22	6.28± 0.19	-0.06
		19	7.4	7.54± 0.3	-0.14
		20	7.13	7.15± 0.55	-0.02
		21	7.33	6.83± 0.54	0.5
		22	7.12	7.03± 1.15	0.09
		23	7.34	7.18± 0.38	0.17
		24	7.81	7.7± 0.63	0.11
		25	7.4	7.06± 0.43	0.34
		26	7.1	7.26± 0.45	-0.16
		27	7.2	7.28± 0.05	-0.08
		28	7.11	7.03± 0.1	0.08
		29	7.37	7.29± 1.3	0.07
		30	7.04	6.84± 0.52	0.2
		31	7.61	7.05± 0.19	0.57

BHI Concentration	Sanitizer Concentration	Isolate	Control (0ppm) Log10 CFU/coupon	Log10 CFU/coupon	Log10 Decrease from Control
Quaternary Ammonium Compound					
1x	100ppm	Average	7.45	7.18±0.56	0.27
		1	7.55	7.62± 1.8	-0.07
		2	8.24	8.06± 0.06	0.17
		3	8.28	7.98± 0.15	0.3
		4	7.15	7.07± 1.06	0.08
		5	8.59	8.1± 0.52	0.49
		6	8.67	8.14± 0.45	0.52
		7	6.36	5.85± 0.93	0.51
		8	8.49	7.85± 0.15	0.64
		9	7.89	7.61± 0.09	0.28
		10	7.73	6.02± 3.08	1.71
		11	7.47	7.43± 0.46	0.03
		12	7.34	7.34± 0.57	0
		13	6.96	6.88± 0.62	0.08
		14	7.3	6.87± 0.39	0.43
		15	7.23	6.87± 0.48	0.37
		16	7.39	6.83± 1.06	0.56
		17	7.16	7.12± 0.55	0.04
		18	6.22	6.2± 0.48	0.02
		19	7.4	7.32± 0.25	0.08
		20	7.13	7.33± 0.47	-0.2
		21	7.33	7.19± 0.59	0.14
		22	7.12	6.91± 0.96	0.21
		23	7.34	7.1± 0.45	0.24
		24	7.81	7.78± 0.56	0.04
		25	7.4	6.67± 0.58	0.73
		26	7.1	7.1± 0.26	0
		27	7.2	7.24± 0.16	-0.05
		28	7.11	7.07± 0.13	0.04
		29	7.37	6.91± 0.93	0.46
		30	7.04	6.93± 0.46	0.11
		31	7.61	7.17± 0.04	0.44

BHI Concentration	Sanitizer Concentration	Isolate	Control (0ppm) Log10 CFU/coupon	Log10 CFU/coupon	Log10 Decrease from Control
Quaternary Ammonium Compound					
1x	200ppm	Average	7.45	7.03±0.58	0.42
		1	7.55	6.85± 1.78	0.7
		2	8.24	7.45± 0.05	0.79
		3	8.28	8.08± 0.17	0.2
		4	7.15	6.94± 1.45	0.21
		5	8.59	8.16± 0.09	0.43
		6	8.67	7.93± 0.34	0.74
		7	6.36	6.11± 1.34	0.25
		8	8.49	7.82± 0.11	0.67
		9	7.89	7.34± 0.3	0.55
		10	7.73	7.73± 0.67	0
		11	7.47	7.34± 0.51	0.13
		12	7.34	6.9± 0.57	0.45
		13	6.96	6.66± 0.93	0.3
		14	7.3	7± 0.17	0.3
		15	7.23	6.75± 0.91	0.48
		16	7.39	6.55± 0.74	0.84
		17	7.16	6.52± 0.4	0.64
		18	6.22	5.45± 0.56	0.77
		19	7.4	7.1± 0.16	0.3
		20	7.13	7.2± 0.28	-0.07
		21	7.33	6.79± 0.48	0.54
		22	7.12	6.58± 0.92	0.55
		23	7.34	7.35± 0.48	0
		24	7.81	7.48± 0.54	0.34
		25	7.4	6.35± 0.27	1.05
		26	7.1	6.81± 0.29	0.29
		27	7.2	7.11± 0.05	0.09
		28	7.11	6.84± 0.32	0.27
		29	7.37	7.2± 1.29	0.16
		30	7.04	6.64± 0.46	0.4
		31	7.61	6.77± 0.19	0.84
		31	7.61	6.77± 0.19	0.84

BHI Concentration	Sanitizer Concentration	Isolate	Control (0ppm) Log10 CFU/coupon	Log10 CFU/coupon	Log10 Decrease from Control
Sodium Hypochlorite					
1/20x	50ppm	Average	5.94	3.43±1.12	2.51
		1	6.31	3.54± 1.57	2.77
		2	6.75	2.17± 2.04	4.58
		3	5.96	1.22± 2.11	4.74
		4	6.01	4.03± 2.14	1.97
		5	6.27	4.72± 1.43	1.56
		6	6.4	4.13± 2.4	2.27
		7	5.55	2.4± 0.66	3.15
		8	5.26	2.2± 2.11	3.06
		9	6.08	3.55± 2.18	2.54
		10	6.08	3.05± 3.01	3.03
		11	5.72	4.67± 1.96	1.05
		12	5.21	4.21± 1.85	1
		13	6.33	5.36± 1.47	0.98
		14	5.57	3.33± 0.55	2.24
		15	6.38	5.2± 1.55	1.19
		16	6.35	2.49± 2.97	3.86
		17	6.04	5.53± 0.52	0.51
		18	6.65	5.02± 0.82	1.63
		19	5.38	1.72± 1.51	3.67
		20	5.62	3.46± 0.45	2.16
		21	6.14	4.17± 1.81	1.97
		22	5.49	2.76± 0.65	2.73
		23	6.02	3.44± 1.24	2.58
		24	6.13	2.77± 2.43	3.35
		25	5.8	3.42± 1.47	2.38
		26	5.68	3.41± 3.37	2.27
		27	6.15	3.79± 2.58	2.35
		28	5.66	1.85± 1.63	3.8
		29	6.18	3.12± 2.46	3.06
		30	5.79	1.99± 3.45	3.8
		31	5.42	3.6± 0.71	1.82

BHI Concentration	Sanitizer Concentration	Isolate	Control (0ppm) Log10 CFU/coupon	Log10 CFU/coupon	Log10 Decrease from Control
Sodium Hypochlorite					
1/20x	100ppm	Average	5.94	2.67±1.07	3.27
		1	6.31	3.15± 2.9	3.16
		2	6.75	3.63± 1.77	3.12
		3	5.96	1.12± 1.94	4.84
		4	6.01	2.88± 2.05	3.13
		5	6.27	3.83± 1.63	2.45
		6	6.4	3.02± 3.09	3.38
		7	5.55	1.7± 1.5	3.84
		8	5.26	1.33± 2.31	3.92
		9	6.08	3.61± 1.82	2.47
		10	6.08	3.5± 1.56	2.58
		11	5.72	3.97± 3.54	1.76
		12	5.21	2.77± 3.23	2.44
		13	6.33	2.8± 3.27	3.54
		14	5.57	1.8± 1.56	3.77
		15	6.38	4.4± 1.31	1.98
		16	6.35	2.22± 2.33	4.13
		17	6.04	3.64± 2.3	2.4
		18	6.65	4.65± 0.83	2
		19	5.38	2.22± 0.54	3.16
		20	5.62	1.23± 1.08	4.39
		21	6.14	4.02± 3.58	2.12
		22	5.49	1.01± 1.74	4.48
		23	6.02	0.93± 1.6	5.09
		24	6.13	2.13± 1.93	4
		25	5.8	3.19± 0.31	2.61
		26	5.68	3.41± 3.42	2.27
		27	6.15	3.07± 2.69	3.08
		28	5.66	1.83± 1.65	3.83
		29	6.18	2.48± 2.19	3.7
		30	5.79	1.18± 2.05	4.61
		31	5.42	2.03± 1.9	3.39

BHI Concentration	Sanitizer Concentration	Isolate	Control (0ppm) Log10 CFU/coupon	Log10 CFU/coupon	Log10 Decrease from Control
Sodium Hypochlorite					
1/20x	200ppm	Average	5.94	2.32±1.10	3.62
		1	6.31	2.59± 3.13	3.72
		2	6.75	0.83± 1.43	5.92
		3	5.96	1.79± 1.57	4.17
		4	6.01	2.1± 3.63	3.91
		5	6.27	4.32± 1.4	1.95
		6	6.4	2.42± 2.65	3.98
		7	5.55	2.67± 0.43	2.88
		8	5.26	0.96± 1.66	4.3
		9	6.08	2.1± 2.07	3.98
		10	6.08	1.9± 1.66	4.18
		11	5.72	3.36± 3.03	2.37
		12	5.21	3.96± 1.78	1.25
		13	6.33	2.7± 2.93	3.63
		14	5.57	0.8± 1.38	4.77
		15	6.38	3.01± 2.93	3.37
		16	6.35	1.74± 3.02	4.61
		17	6.04	3.14± 2.78	2.9
		18	6.65	3.84± 1.62	2.8
		19	5.38	2.16± 0.45	3.22
		20	5.62	1.42± 2.46	4.2
		21	6.14	2.54± 2.25	3.6
		22	5.49	2.21± 2.03	3.28
		23	6.02	0.87± 1.5	5.15
		24	6.13	3.72± 1.53	2.4
		25	5.8	1.01± 1.74	4.8
		26	5.68	3.68± 3.28	2
		27	6.15	2.62± 2.57	3.53
		28	5.66	1.54± 1.47	4.11
		29	6.18	3.97± 1.5	2.21
		30	5.79	1.91± 3.31	3.87
		31	5.42	0± 0	5.42

BHI Concentration	Sanitizer Concentration	Isolate	Control (0ppm) Log10 CFU/coupon	Log10 CFU/coupon	Log10 Decrease from Control
Sodium Hypochlorite					
1x	50ppm	Average	7.08	5.62±0.56	1.46
		1	6.7	4.95± 1.99	1.75
		2	6.58	4.92± 1.48	1.66
		3	7.2	5.46± 1.91	1.74
		4	6.97	5± 0.49	1.97
		5	6.98	5.92± 1.05	1.06
		6	7.1	5.91± 1.04	1.19
		7	6.61	4.95± 0.93	1.66
		8	7.29	6.04± 0.55	1.25
		9	7.55	5.91± 0.85	1.64
		10	7.13	5.98± 1.37	1.15
		11	6.65	5.72± 0.42	0.93
		12	7.28	5.24± 0.45	2.03
		13	6.71	4.7± 1.42	2
		14	6.8	5.76± 1.56	1.04
		15	7.2	5.27± 0.69	1.93
		16	7.16	5.99± 0.25	1.16
		17	7.06	5.26± 0.92	1.8
		18	6.73	4.73± 2.05	1.99
		19	7.11	6.38± 0.67	0.73
		20	7.37	6.36± 0.72	1.01
		21	6.95	5.14± 0.68	1.8
		22	7.22	5.39± 0.21	1.83
		23	7.28	6.65± 0.41	0.63
		24	7.1	5.98± 1.11	1.12
		25	7.06	4.95± 1.03	2.1
		26	6.95	5.15± 1.93	1.8
		27	7.46	6.4± 1.03	1.06
		28	7.04	6.34± 0.32	0.7
		29	7.53	5.96± 1.01	1.57
		30	7.41	6.28± 1.14	1.13
		31	7.37	5.38± 0.93	2

BHI Concentration	Sanitizer Concentration	Isolate	Control (0ppm) Log10 CFU/coupon	Log10 CFU/coupon	Log10 Decrease from Control
Sodium Hypochlorite					
1x	100ppm	Average	7.08	5.00±0.66	2.08
		1	6.7	4.86± 1.58	1.84
		2	6.58	4.41± 2.09	2.17
		3	7.2	3.93± 1.11	3.27
		4	6.97	4.29± 0.22	2.68
		5	6.98	4.57± 1.04	2.41
		6	7.1	6.26± 0.67	0.84
		7	6.61	4.98± 1.25	1.63
		8	7.29	5.2± 1.04	2.09
		9	7.55	4.69± 0.62	2.86
		10	7.13	5.35± 0.86	1.78
		11	6.65	4.54± 0.82	2.11
		12	7.28	5.28± 1.31	1.99
		13	6.71	3.83± 0.44	2.88
		14	6.8	4.87± 0.74	1.93
		15	7.2	3.87± 1.38	3.33
		16	7.16	5.19± 0.94	1.96
		17	7.06	5.18± 0.96	1.87
		18	6.73	4.24± 1.88	2.49
		19	7.11	5.27± 0.67	1.84
		20	7.37	6.15± 0.96	1.22
		21	6.95	4.62± 0.76	2.32
		22	7.22	5.99± 0.45	1.22
		23	7.28	5.36± 0.52	1.92
		24	7.1	6.06± 0.56	1.04
		25	7.06	5.37± 1.63	1.69
		26	6.95	5.5± 1.12	1.45
		27	7.46	4.51± 1.65	2.95
		28	7.04	4.41± 0.47	2.63
		29	7.53	5.78± 0.8	1.75
		30	7.41	5.35± 1.57	2.07
		31	7.37	5.1± 0.65	2.27

BHI Concentration	Sanitizer Concentration	Isolate	Control (0ppm) Log10 CFU/coupon	Log10 CFU/coupon	Log10 Decrease from Control
Sodium Hypochlorite					
1x	200ppm	Average	7.08	4.48±0.72	2.6
		1	6.7	5± 0.84	1.7
		2	6.58	4.37± 1.47	2.21
		3	7.2	3.46± 2.2	3.74
		4	6.97	4.62± 1.24	2.35
		5	6.98	3.85± 0.88	3.14
		6	7.1	4.7± 1.61	2.4
		7	6.61	4.8± 0.39	1.81
		8	7.29	5.19± 1.74	2.1
		9	7.55	5.11± 0.86	2.45
		10	7.13	4.77± 1.35	2.36
		11	6.65	4.57± 0.85	2.08
		12	7.28	4.4± 0.83	2.87
		13	6.71	3.03± 0.35	3.67
		14	6.8	4.79± 0.44	2.01
		15	7.2	3.57± 1.21	3.63
		16	7.16	4.01± 1.75	3.14
		17	7.06	4.18± 0.54	2.87
		18	6.73	2.82± 3	3.91
		19	7.11	5.8± 0.42	1.31
		20	7.37	4.38± 0.47	2.98
		21	6.95	4.6± 0.56	2.34
		22	7.22	4.13± 1.14	3.09
		23	7.28	4.41± 0.45	2.87
		24	7.1	5.06± 0.16	2.05
		25	7.06	3.93± 1.53	3.13
		26	6.95	4.14± 1.01	2.82
		27	7.46	5.14± 1.47	2.31
		28	7.04	4.56± 0.24	2.48
		29	7.53	5.63± 0.37	1.91
		30	7.41	5.88± 0.35	1.54
		31	7.37	4.01± 0.27	3.37

Figures

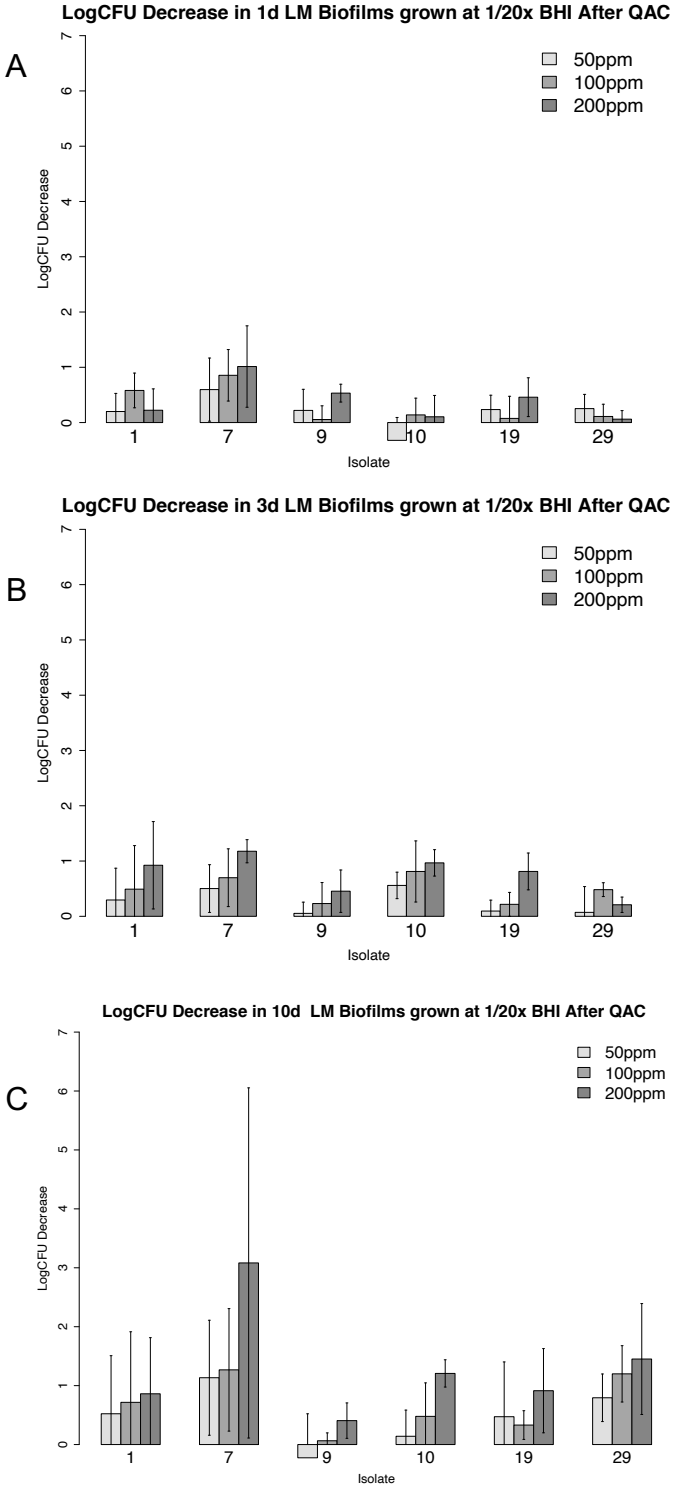

Figure 1: Quaternary ammonium compound treatment of *L. monocytogenes* biofilms at nutrient poor conditions. Decrease in LogCFU per coupon after QAC treatment on *L. monocytogenes* biofilms A.1, B. 3, and C. 10 days post incubation in 1/20x BHI. Error bars represent standard deviation between experimental replicates.

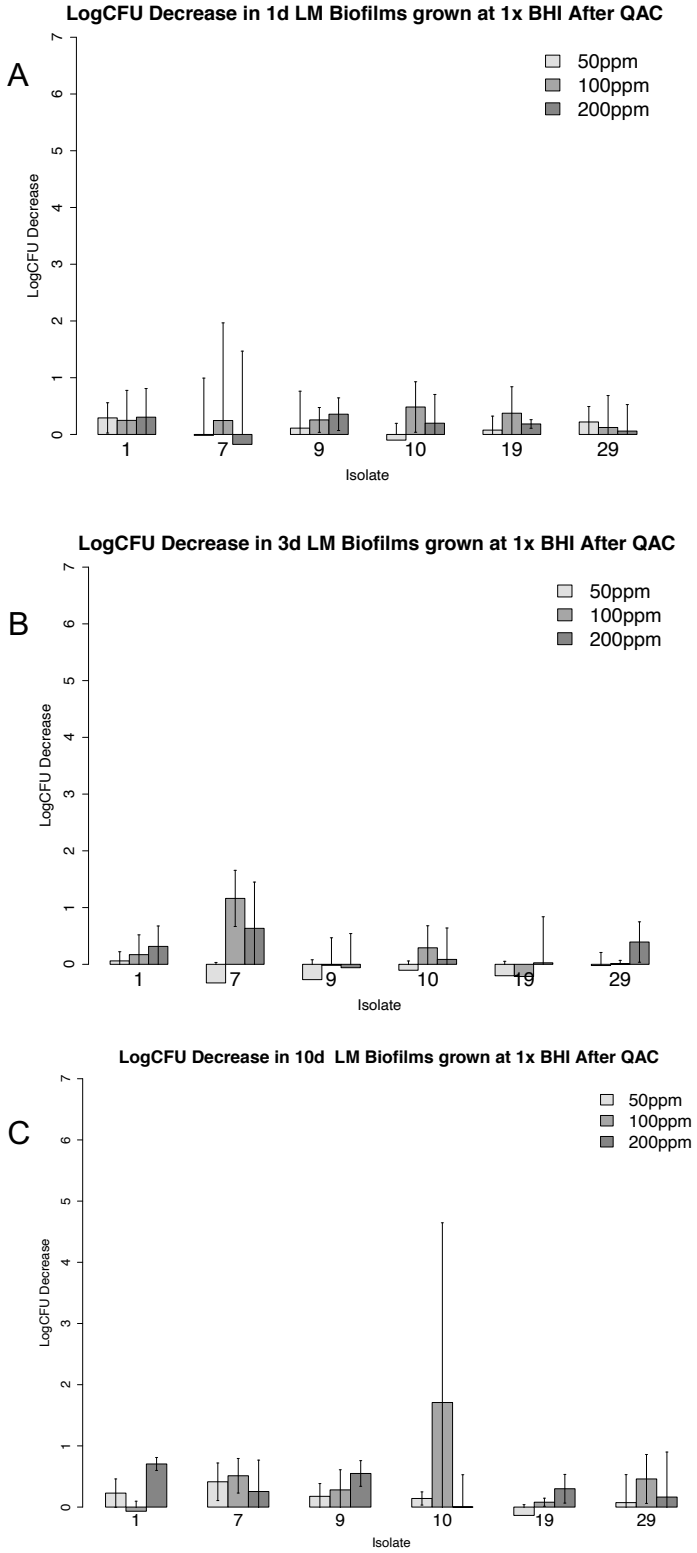

Figure 2:Quaternary ammonium compound treatment of *L. monocytogenes* biofilms at nutrient rich conditions. Decrease in LogCFU per coupon after QAC treatment on *L. monocytogenes* biofilms A.1, B. 3, and C. 10 days post incubation in 1x BHI. Error bars represent standard deviation between experimental replicates.

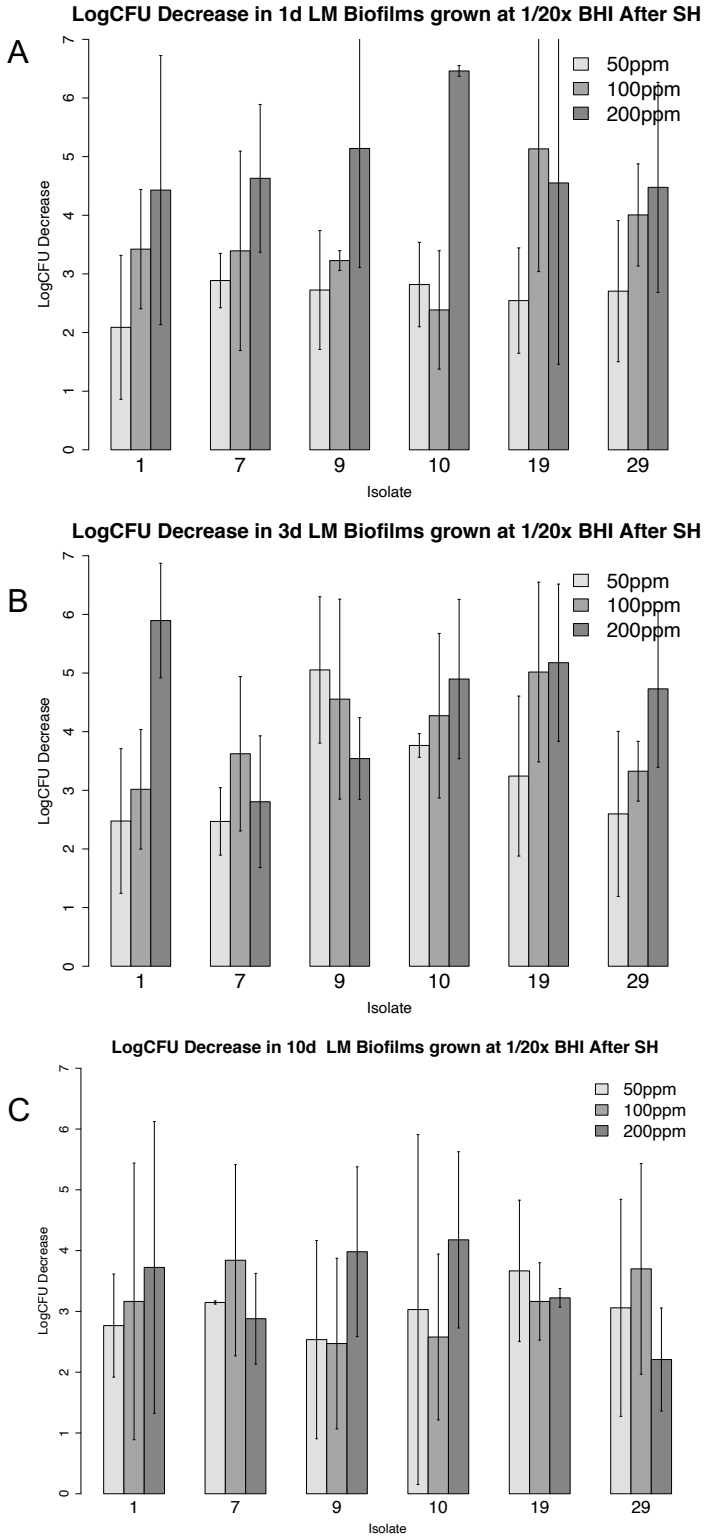

Figure 3: Sodium hypochlorite treatment of *L. monocytogenes* biofilms at nutrient poor conditions. Decrease in LogCFU per coupon after SH treatment on *L. monocytogenes* biofilms A.1, B. 3, and C. 10 days post incubation in 1/20x BHI. Error bars represent standard deviation between experimental replicates.

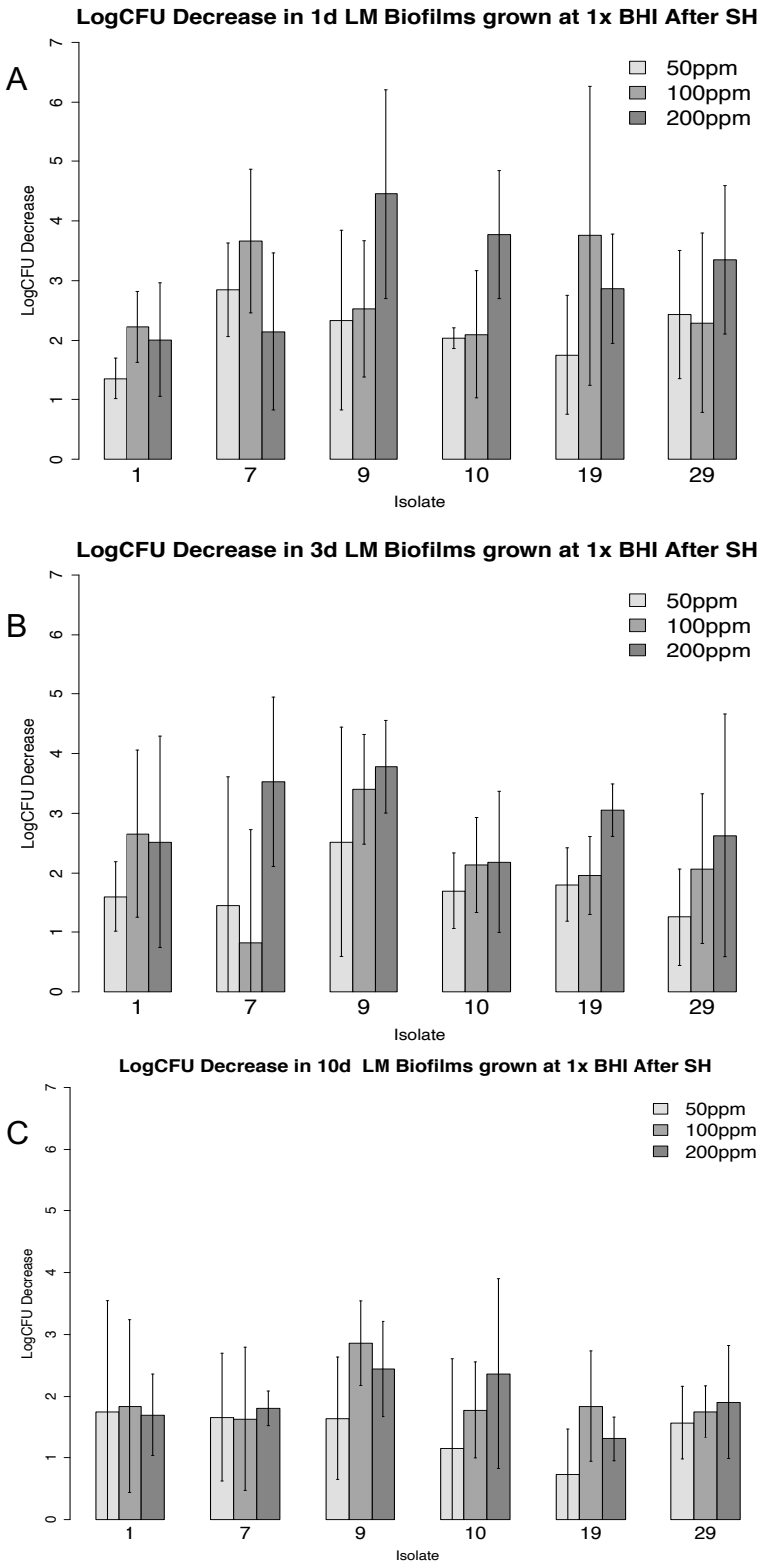

Figure 4: Sodium hypochlorite treatment of *L. monocytogenes* biofilms at nutrient rich conditions. Decrease in LogCFU per coupon after SH treatment on *L. monocytogenes* biofilms A.1, B. 3, and C. 10 days post incubation in 1x BHI. Error bars represent standard deviation between experimental replicates.

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