Effect of Delmopinol Hydrochloride on the Prevention and Removal of *Listeria monocytogenes* and *Salmonella enterica* Stainless Steel-Adhered Biofilms.

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ABSTRACT

Bacterial biofilms attached to food contact surfaces are an ongoing concern for the food industry due to the resistance of bacteria within biofilms to detergents and sanitizers. Within food manufacturing facilities, stainless steel is a common food-contact surface in which microbial cell attachment and biofilm formation may occur. Identifying methods to prevent and remove biofilms during standard cleaning and sanitation practices could prove useful, as mature biofilms can release planktonic cells into an aqueous environment, causing continual low-level contamination. Dental studies involving delmopinol hydrochloride, a cationic surfactant, have found a preventative and dissociating affect on biofilms, where food applications have scarcely been researched.

This study demonstrates the prevention and removal of *Listeria monocytogenes* 1/2a and *S. enterica* Agona biofilms on stainless steel with pre- and post-exposures of delmopinol hydrochloride. Stainless steel blanks (#304, 16 gauge, 2cm x 2cm, finish #4) were submerged in a 0.2% or 0.5% delmopinol solution before or after biofilm formation. Treatment times were 1, 5 or 10 minutes, whereas controls were not exposed to the delmopinol solution. Disinfected stainless steel blanks were spot-inoculated with 20µL of a 10^9 CFU/mL liquid culture, and pre-exposed blanks were additionally submerged in delmopinol and dried prior to inoculation. Biofilms were exclusively formed on the finished and inoculated side by placing the surface face-down on TSA. After cell attachment and biofilm development for 24 hours at 25°C, blanks were rinsed with phosphate buffer. Post-exposed blanks were submerged in 0.2% or 0.5% delmopinol for 1, 5 or 10 minutes before all blanks were individually vortexed for 90 seconds to

dislodge films. Bacterial populations were determined by surface plating onto TSA followed by incubation at 32ºC for *L. monocytogenes* and 37ºC for *S.* Agona for 48 hours. Treatments were in-duplicate and repeated three times for each microorganism.

Pre-exposure of 0.2% delmopinol resulted in a significant decrease in *L. monocytogenes* concentration at 1, 5 and 10 minute exposures ($P < 0.05$). Pre-exposures with the 0.5% solution had no significant effect on *L. monocytogenes* biofilm populations ($P > 0.05$), whereas all post-exposures lead to a significant decline in biofilm concentrations ($P < 0.0001$). Post-exposures of 10 minutes exhibited a mean $\log_{10}$ reduction of 5.59 and 6.40 $\log_{10}$ for 0.2% and 0.5% delmopinol solutions, respectively. For *S.* Agona, 0.2% pre-exposure resulted in no significant $\log_{10}$ reduction ($P > 0.05$), while the 10 minute 0.5% pre-exposure exhibited a minimal reduction in bacterial growth ($P < 0.05$). Post-exposures of 10 minutes exhibited a mean $\log_{10}$ reduction of 7.65 and 7.75 $\log_{10}$ for 0.2% and 0.5% delmopinol solutions, respectively. For *L. monocytogenes* and *S.* Agona, post-exposure to delmopinol hydrochloride caused a notable $\log_{10}$ reduction. The removal effect of delmopinol on biofilms is significantly greater the preventative effect.

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Dedication

Dedicated to my closest loved ones, namely Emily S. Ewell, Marcy E. Cathey, Gregory S. Ewell, Jill R. Ewell, Aaron Frank, Lily L. Yang and J. Alexander Potter.

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CHAPTER I

INTRODUCTION

Cleanliness and sanitation are top priorities in the food industry. Adequate cleaning removes food debris, residue and other soils with the goal of creating clean surfaces suitable for sanitation with a chemical sanitizer. Despite the application of cleaning and sanitization procedures in food manufacturing plants, bacterial biofilms containing food pathogens are not always effectively removed from food-contact surfaces (Cox, 1989). Through the production of exopolysaccharide films (Zottola, 1991), bacteria embedded within a biofilm are naturally resistant to chemical sanitizers (Ronner, 1993). Sodium hypochlorite, among other sanitizers, can effectively reduce bacterial populations with proper exposure time and solution concentration whether bacteria are planktonic or protected by a biofilm, however a higher concentration and contact time is essential to produce cell death when protected by a biofilm (Lee, 1991).

Biofilms formed on food-contact surfaces, such as plastic, stainless steel and polytetrafluoroethylene (Teflon®), may be responsible for instances of post-process contamination through the release of embedded bacteria to become planktonic and colonize food after contact. *Listeria monocytogenes* is of special concern in certain food products because of the ability of this microorganism to grow at refrigeration temperatures, allowing for low bacterial levels to proliferate to an infectious concentration despite a proper food storage temperature (Gray, 1966). Heat is the most effective method for eliminating viable biofilm-embedded bacteria, although many processing facilities are unable to apply water with temperatures required to cause rapid cell death (Chmielewski, 2004).

Stainless steel, in varying grades and finishes, is the most utilized food-contact surface in food manufacturing facilities due to the corrosion resistance, durability and affordability of the

material. (Spragg, 1977). *L. monocytogenes* and *Salmonella* spp. may form biofilms on stainless steel when these microorganisms are allowed to remain on food-contact surfaces as a result of inadequate cleaning and sanitation. (Cox, 1989). Furthermore, these pathogens may cause moderate to severe foodborne illness when ingested by vulnerable individuals through contaminated food. The natural chemical sanitizer and preservation resistance of microorganisms within a biofilm creates the need for alternative sanitation measures. Consequently, the ability to prevent and dissolve biofilms on food-contact and plant surfaces is of great concern.

Delmopinol hydrochloride, often marketed as decapinol, is a cationic surfactant with the ability to interact with biofilms. The compound was developed for dental applications to prevent and remove oral bacteria biofilms on gums and tooth enamel (Sunstar, 2013). Since its incorporation into various commercial products, including mouthwash, sprays and lozenges, delmopiniol hydrochloride has been highlighted in two food pathogen studies evaluating effectiveness in diminishing *Campylobacter* adhesion on food-contact surfaces and *Salmonella* Michigan biofilms on cantaloupe rind surfaces (Saucedo, 2013; Waldron, 2013). The concentration required for effectiveness is relatively low; 0.2% (w/w) is used commercially and up to 1.0% (w/w) has been used in food pathogen studies. Results indicate that delmopinol could serve as a potential industrial surfactant capable of degrading and possibly preventing foodborne pathogens on stainless steel and other food contact surfaces.

Studies have demonstrated how the gram-stain, or more specifically, cell wall surface structure and abundance of peptidoglycan of a microorganism, might influence how well biofilms are prevented and dislodged by delmopinol. *Salmonella* spp. (nontyphoidal) are the most common gram-negative bacteria causing foodborne infection, while *L. monocytogenes* is the most fatal gram-positive foodborne bacteria within the United States (CDC, 2012c). While

there have been several outbreaks of *Listeria monocytogenes* in the United States, food product recalls are more common due to the presence of *L. monocytogenes* in contaminated food products. Listeriosis is rare in healthy individuals, where infection opportunistically occurs in pregnant women, infants, the elderly and the immunodeficient. With a current case fatality of approximately 16.25%, pregnant women are likely to experience a stillbirth or miscarriage if infected (Scallan, 2011). *S. enterica* causes more illnesses and deaths than *L. monocytogenes* each year, where there are an estimated 1,000,000 infections, 19,000 hospitalizations and nearly 400 deaths annually (CDC, 2012a).

Given the threat of biofilm development within a food processing facility paired with the difficulty of removing biofilms from surfaces once formed, compounds capable of biofilm prevention and dissociation would be beneficial to the food industry through the addition to standard cleaning practices. Cleaning removes debris, while the inclusion of an additional step between cleaning and sanitation could prevent future contamination by dissolving the biofilm matrix, allowing for previously bound bacteria to be adequately eradicated by a subsequent sanitizer. Evaluating the effectiveness of delmopinol hydrochloride on the preventative and dissociating nature of food pathogen biofilms is thus an advantage to both the food industry and the compound manufacturer, as the possibilities to extend applications beyond dental biofilms holds great potential.

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CHAPTER II

LITERATURE REVIEW

Foodborne Pathogens in the United States

Potential contamination of food products with foodborne microorganisms that are pathogenic to humans is a threat to manufacturers and consumers of processed foods and produce, where the bacterial load of both foods and facilities must be monitored. The ability to prevent contamination and decrease microbial load through good manufacturing practices (GMPs), good agricultural practices (GAPs) and facility sanitation is highly valued. Despite the ideal where every facility and distributor meets sanitary guidelines, contamination and poor bacterial reduction may still occur.

The Center for Disease Control (CDC) continually compiles data related to foodborne illnesses to monitor trends related to factors causing illness, as well as the prevalence of various microorganisms. Currently, 48 million individuals contract a foodborne illness within the United States annually. Over 128,000 of these individuals are admitted to the hospital, while 3,000 die from infection (CDC, 2012a). Among these 48 million individuals, it is estimated that approximately 9.4 million cases are confirmed to be caused by 31 known pathogens including bacteria, parasites and viruses. While most foodborne illnesses are caused by Norovirus (58%), *Salmonella* spp. are known to cause the second-most confirmed illnesses at 11%. Other microorganisms within the top five foodborne pathogens include *Clostridium perfringens* (10%), *Campylobacter* spp. (9%) and *Staphylococcus aureus* (3%). Due to the widespread distribution of *Salmonella* cells causing infection, this genus also causes the most hospitalizations (35%) and deaths (28%). Although rare, *Listeria monocytogenes* is a threat to public health due to a high fatality rate, accounting for 19% of foodborne-related deaths annually in the United States (CDC,

2012a). The incidence of bacterial infections based on laboratory-confirmed cases, as of 2012, is also monitored, as this data is useful in prioritizing emerging pathogens as well as tracking the efficiency of current regulations and methods aimed at decreasing infections. Since 2006-2008 within the United States, *Campylobacter* spp. and *Vibrio* spp. confirmed cases are increasing, while *L. monocytogenes*, *Shigella* spp., *Escherichia coli* STEC O157 and *Yersinia* spp. are experiencing a decline, hinting at effectiveness despite a lack of statistical significant difference. There has been no detectable change in confirmed *Salmonella* spp. illnesses within the United States, thus serving as a continuing threat to public health (CDC, 2012c).

Listeria monocytogenes

The *Listeria* genus consists of gram-positive, rod-shaped and facultatively anaerobic bacteria with seven species, including *L. monocytogenes, L. ivanovii, L. murrayi, L. innocua, L. welshimeri, L. grayi* and *L. seeligeri. Listeria monocytogenes* exhibits tumbling motility via peritrichous flagella below 30°C, despite optimal growth between 30°C and 37°C (Todar, 2008). Within the *Listeria* genus, *L. monocytogenes* has been found to cause listeriosis in humans. Analyzing all 13 serovars of *L. monocytogenes*, only three are commonly associated with foodborne illness including 1/2a, 1/2c and 4b (Ward, 2004). With relation to human infections, 4b is traditionally included in research, as 33 to 50% of human cases (internationally) consist of this serovar (Borucki, 2003). Comparatively, 1/2a strains are the second-most common serovar isolated from listeriosis cases (Dharmarha, 2009).

L. monocytogenes is generally associated with post-processing contamination (McLauchlin, 1987), and has been isolated from drains, still water, residues, food-contact surfaces and floors in food processing plants (Cox, 1989). Outbreaks of listeriosis have been

associated with soft cheeses (Pini, 1988), fish (Eklund, 1995), vegetables (Breer, 1992), ready-to-eat meats (Gilbert, 1993) and milk (Harvey, 1992). Human exposure to *L. monocytogenes* is commonly the result of contaminated food, as food is the primary vehicle of transmission (Farber, 1991). *Listeria monocytogenes* is especially troublesome to the food industry given the tolerance of this microorganism to preservatives (Shahamat, 1980) and the ability of the organism to grow at the refrigeration temperature of 4°C (Gray, 1966).

Listeriosis and *Listeria monocytogenes* Outbreaks

Listeriosis is an infection which may result from the consumption of contaminated food. *L. monocytogenes* is an opportunistic pathogen, infecting immunocompromised consumers, including pregnant women, young children, the elderly and individuals with weakened immune systems as a result of an illness or disease, such as AIDS. Once infected, listeriosis causes fever, followed by muscle aches and gastrointestinal distress. Most infections become invasive, allowing for bacteria to infect a host beyond the gastrointestinal tract, where pregnant women may experience a miscarriage, premature delivery, newborn infection or stillbirth (CDC, 2012b).

L. monocytogenes has been indentified in outbreaks of foodborne illness throughout the United States, with approximately 1600 to 2500 listeriosis cases annually (CDC, 2012b; FDA, 2009). While *L. monocytogenes* does not cause as many foodborne illnesses as *Salmonella* strains or *E. coli*, the mortality rate of listeriosis is roughly 16.25% (Scallan, 2011). For this reason, listeriosis comprises a relatively small amount of foodborne illnesses in the US annually (0.02%), but a surprising 27.6% of all foodborne infection deaths (FDA, 2009).

Since 1970, the Centers for Disease Control and Prevention (CDC) has recorded seven of listeriosis in the United States. During 1983, in one of the first recorded foodborne outbreaks of

listeriosis, 14 deaths were associated with consumption of contaminated pasteurized whole and 2% milk (CDC, 1990). In 1994 four hospitalizations were also associated with *Listeria*-contaminated pasteurized milk (Dalton, 1997). The third and most-deadly outbreak was also related to dairy products and took place in California, where contaminated queso blanco lead to 52 deaths, including 19 stillbirths and 10 infant deaths. Since 1998, *L. monocytogenes* has also been associated with ready-to-eat (RTE) meats, as an outbreak within that year associated with contaminated hot dogs and cold cuts caused 14 to 21 deaths, including 4 miscarriages (Neuman, 2011).

Despite an increased understanding of *L. monocytogenes* and listeriosis, recalls attributed to contaminated produce have become more prominent. Among multiple salad recalls within the past few years, the summer of 2011 brought the second-most deadly outbreak in the United States, where *L. monocytogenes* infections caused 30 recorded deaths after consumption of contaminated cantaloupe (CDC, 2011). This outbreak specifically heightened the concern for contamination of produce such as leafy greens, sprouts, celery and cantaloupe with *L. monocytogenes* (CDC, 2013).

Listeria monocytogenes outbreak trends indicate that microbial contamination occurs after food has been processed, such as pasteurization for dairy, cooking for RTE meats. Improperly cleaned and sanitized food contact surfaces, such as stainless steel, are suitable for biofilm formation, where it is likely that planktonic bacteria free within a liquid environment may leave biofilm communities, resulting in post-process contamination and subsequent foodborne illnesses.

Salmonella spp.

The *Salmonella* genus consists of gram-negative, rod-shaped and flagellated bacteria that are aerobic to facultatively anaerobic. There are two species, *S. enterica* and *S. bongori*, where *S. enterica* is the most prevalent specie, containing over 2,500 serovars. The bacterial reservoir is the intestinal tracts of animals, with a high association with poultry, cattle, pigs and sheep (Montville, 2008). Produce-related outbreaks indicate a fecal source of infection, where specific sources may be attributed to agricultural animals that have contaminated water sources. Environmentally, *Salmonella* is able to live for a significant period of time outside of an organism, where growth and the ability to survive depend mainly on the availability of water and nutrients. The ability of *S. enterica* to produce biofilms paired with its environmental stability makes this organism a particularly difficult pathogen to effectively control (Cavallaro, 2011). It is likely that produce may be exposed by *Salmonella* through contaminated soil and/or water.

Salmonellosis and *Salmonella enterica* Outbreaks

Nontyphoidal *Salmonella enterica* account for the majority of bacterial foodborne illness in the United States each year (CDC, 2012a), where the number of cases typically increases or remains constant each year (Montville, 2008). Outbreaks have been related to a varying types of foods including produce, poultry, eggs and products containing eggs, dairy, spices and peanuts.

Repeated, notable outbreaks of *Salmonella enterica* serovar Enteritidis have been associated with shell eggs. Many salmonellosis outbreaks result after improper heat-related bacterial reduction procedures relative to the consumer, improper sanitation and temperature abuse related to cooling and holding periods, where a product can further contaminate foods if incorporated into multiple products. Poor sanitation and ingredient contamination was evident in

a U.S. outbreak where the tanker used to transport unpasteurized liquid eggs later held a pasteurized liquid mixture destined for ice cream production without being properly sanitized. With an attack rate of 6.6%, this ultimately lead to a 224,000 cases of *S.* Enteritidis outbreak infection (Hennessy, 1996; Montville, 2008). Poor holding temperatures and unhygienic conditions were attributed to a peanut paste associated outbreak in 2008-2009 of *S.* Typhimurium, where improper ventilation and poorly implemented good manufacturing practices (GMPs) led to contaminated product. As a large distributor of peanut products for various food manufacturers, the *Salmonella* contamination lead to a 46-state, 714-case outbreak that included 9 deaths (Cavallaro, 2011).

Salmonella spp. outbreaks have more recently been attributed to low-moisture, high-fat foods. This includes chocolate, peanut butter and potato chips, all foods that were initially thought to both prevent growth and discourage cell survival as a result of low water activity (Podolak, 2010). These outbreaks show that viable *Salmonella* survive in dry environments, such as surfaces in food plants after contact with contaminated or raw products. The ability to survive in dry environments is what has typically lead to fresh produce contamination following soil exposure, where the spread of bacteria can lead to biofilms on both produce and food-contact surfaces within a manufacturing environment (Cavallaro, 2011).

Biofilm Forming Capacity in *L. monocytogenes* and *Salmonella enterica*

The "forming capacity" of biofilms is commonly evaluated through reading the optical density (OD_{595}) of crystal violet-stained biofilms in microtiter plates. Microtiter plate wells are filled with inoculated liquid media, where bacteria are allowed to grow within the individual wells, leading to cell attachment to the plate polymer, namely polystyrene, followed by biofilm

development. The growth medium is discarded after incubation and the wells are rinsed with sterile, distilled water to remove unattached cells. The remaining biofilms are stained with crystal violet and lightly de-stained with ethanol before the optical density is read. After adjusting for non-biofilm control wells, the optical density values are used to determine the quantity of biofilm formed relative to other treatment wells, where the higher the value the thicker the biofilm that was produced (Borucki, 2003; Vestby, 2009).

L. monocytogenes serovar 1/2c has been found to adhere more readily to stainless steel than 4b, with 1/2a being the least adherent (Norwood, 1999). To some extent this conflicts with Borucki et al 2003, which found two 1/2a serovars and a 4b serovar that created a more stable biofilm than many 1/2c serovars, although, on average, 1/2c out-performs most 4b and 1/2a serovars in this regard.

Biofilms and the Food Industry

The food industry has adopted effective methods of cleaning and sanitation in order to prevent most bacterial forms of contamination during processing. Food debris is removed before sanitizers (such as sodium hypochlorite or ammonium quaternary compounds) are applied to a surface, as this is a generally effective measure of decreasing or eliminating microorganisms. An exception arises when biofilms are present, as multispecies biofilms are quite common on food-contact surfaces, especially within the dairy industry (Blaschek, 2007). Biofilms may form on stainless steel, glass, plastics, rubber and polytetrafluoroethylene (PTFE), among other surfaces (A. B. Ronner, A.C.L. Wong, 1993). The more hydrophobic a surface, the more likely a biofilm will form, as the repellence of water increases the ability for bacteria to adhere securely to the surface. Non-stick materials, as with PTFE, also commonly known as Teflon®, are most

susceptible for biofilm formation. Rubber gaskets and seals are often not replaced with a frequency that prevents bacterial growth, as cracks formed within rubber equipment allow for growth via a protective pocket often untouched by detergents and sanitizers (Parkar, 2004). These conditions allow for spoilage and pathogenic bacteria to proliferate, forming multispecies biofilms. Specific to dairy processing facilities, *Pseudomonas* species, *Listeria monocytogenes* and *Salmonella enterica* serovars are most likely to form these multispecies communities. *Pseudomonas* species generally cause milk spoilage, while *Salmonella* may cause foodborne illness. Additionally, *L. monocytogenes* is a notable threat, as this pathogen causes severe illness and death (Blaschek, 2007; Brooks, 2008).

Biofilms are comprised of a sessile community of bacteria surrounded by a matrix composed of extracellular polymeric substances (EPS; also known as exopolysaccharides). EPS itself is comprised of extracellular DNA, polysaccharides and proteins, forming a "slime-like" substance in which bacteria may implant themselves (Blaschek, 2007). The structure of the EPS and sessile bacteria is not homogeneous, and differs between the surface to which bacteria have irreversibly attached and the surrounding environment. Bacteria exude this film to a thickness of a few micrometers to several millimeters, and despite the fact that a biofilm is 90-97% water, the EPS component serves as a protective layer. The construction of the film acts to store nutrients, allow ample water flow and prevent the infiltration of harmful substances (Sutherland, 2001).

Biofilm formation is a two-step process, bacteria reversibly adhere to a surface via electrostatic forces (hydrogen bonds or Van der Waals forces) then a second irreversible attachment process occurs over time (Marshall, 1971). The production of exopolysaccharide (EPS) occurs during the second step, trapping nutrients and protecting embedded cells (Zottola, 1991). Nutrition deprivation is known to increase biofilm formation, especially in *Listeria*

monocytogenes, as this characteristic of survival in unfavorable environments (Brown, 1977). Once a biofilm has formed, microorganisms become more resistant to sanitizers, but residual bacteria may dislodge from biofilms, allowing for post-process contamination. This is especially evident if biofilms have formed after pasteurization or cleaning procedures (Ronner, 1993). *L. monocytogenes* biofilms have been known to adhere to stainless steel for eight days and longer, where bacteria embedded within a biofilm may become 100 times more resistant to bleach (3-8%) after four days of biofilm development (Lee, 1991). Comparatively, *Salmonella* spp. biofilms on produce and poultry skin surfaces have been studied, where rough plant or skin tissue can harbor bacteria and encourage biofilm growth. The limited ability to sanitize produce or poultry paired with the potential presence of biofilm communities further limits microbial reduction procedures implemented by the produce industry (Annous, 2005; Saucedo, 2013; Waldron, 2013).

Stainless Steel

Austenitic stainless steel (alloyed carbon aligned in face-centered cubic crystal) has been the primary choice for food processing equipment for decades due to the inert nature of this metal paired with corrosion resistance and ease of cleaning (Spragg, 1977). Grades 304 (surgical stainless steel; 18-20% chromium and 8-10% nickel) and 316 are most-common to the food industry, with finishes 2B (mill) and #4 (satin) most-used with the 304 grade variety, where surface roughness (R_a) is less than 1µm (NSF, 2000). Finish #4 is most common in the United States, while 2B is the predominant food processing equipment material in Europe (Boulange-Peterman, 1996). The wettability, *i.e.* the ability of a surface to retain water, of a stainless steel is relative to the finish, where the more hydrophobic a surface, the more likely a biofilm will form.

Sofyan et al 2006 explored the adhesion of *Listeria monocytogenes* to stainless steel of finishes 2B, #4 and #8 (mirror), where attachment was highest among very fine finishes (#8), as the smoothness increases bacterial adhesion and the hydrophobic properties of the steel (Sofyan, 2006). This study concluded that finish 2B harbors the least bacterial count of *L. monoctyogenes* in comparison to #4, and explains why mirror finishes (#8) are uncommon for food-contact surfaces (Sofyan, 2006). This likely applies to other bacteria species during biofilm development.

Delmopinol Hydrochloride

Delmopinol hydrochloride is a relatively new, commercial product compound currently marketed for plaque reduction and control in dental applications. Commercialized mouth rinse, toothpaste, gel and spray products containing the ingredient Decapinol® are available with the active ingredient, delmopinol, at a concentration of 0.2%. Sinclair Pharmaceuticals Limited, a pharmaceutical company based in London, UK and Paris, France, is responsible for the development and production of the compound. Sinclair describes Decapinol® as an "invisible barrier preventing bacteria from adhering to the tooth surfaces", implying that the compound dissolves biofilms and also prevents the adhesion of bacteria and biofilm formation (Sunstar, 2013).

The Food and Drug Administration (FDA) recently approved "Decapinol Oral Rinse – K041482" on April 18, 2005. The rinse is approved for the prevention of gingivitis and periodontitis, and due to the lack of information regarding use by children and pregnant women, it is advised that pregnant women and children under twelve years old avoid using the product (FDA, 2005). In the United States of America, a product containing delmopinol is GUM®

PerioSheild™ Oral Health Rinse, a brand name owned by Sunstar Americas, Incoroporated. This rinse is described as a "next-generation" oral rinse, advertising the reduction of plaque, as well as the prevention of bleeding gums by 36%, as determined by data the company has collected. Suggested use for plaque reduction overtime includes two daily rinses with 10mL of the rinse for 30 seconds. (Sunstar, 2013).

Chemically, delmopinol is paired with hydrochloride to form the International Non-proprietary Name (INN) 3-(4-propylheptyl)-4-morpholinethanol with a molecular weight of 307.90 g/mol. This structure is paired with hydrochloride to create a delmopinol hydrochloride salt, where a 1% (w/v) aqueous solution has a pH range between 4 and 5 (Lundbeck Pharmaceuticals, 2009). The process for producing delmopinol is complex and can follow a variety of chemical reactions, however the U.S. patent (US 7910730 B2) published on March 22, 2011 applied by Sinclair Pharmaceuticals Limited describes the process as "a short and convergent synthesis which takes place through a reaction between oxazolidin[2,3-c]morpholine compound and a Grignard compound." In actuality the process is time-intensive, costly and requires highly toxic reagents, all of which contribute to the current high end-cost. Sinclair Pharmaceuticals is currently developing new, cost-effective measures to produce this biofilm-dissolving and preventing compound (Surroca, 2010).

Delmopinol Hydrochloride Dental Studies

Delmopinol at a concentration of 0.2% was shown to be better than controls for reduction of plaque in a study that met American Dental Association (ADA) criteria (Addy, 2006). *Streptococcus mutans*, a gram-positive bacteria commonly associated with oral bacterial flora, has been shown to be displaced when imbedded within a biofilm, where a glass-adhered biofilm

treated with delmopinol observed under a transmission electron microscope (TEM) exhibited and "empty of unordered matrix" between cells, indicating poor biofilm development and notable removal of cells. As a result, it was found that delmopinol reduced glucan-containing plaque, allowing for further removal of bacteria through mechanical action (Rundegren, 1992).

Many studies have demonstrated the low antimicrobial action of delmopinol, noting that delmopinol has more potential as a surfactant than as a bactericidal agent. (Rundegren, 1992) An artificial mouth system was used in an *in vitro* study to determine the effect of plaque formation, acid production by oral bacteria and salivary bacterial count. The benefit of *in vitro* studies includes enhanced monitoring of plaque in that bacterial counts are more accurate when a surface can be removed from the system in comparison to a human mouth where teeth would not be removed for enumeration (Simonsson, 1991). This has encouraged studies where enameled pieces have been placed within the mouth of a panelist to encourage biofilm growth before a study is conducted. Relative to this early study in 1991, the authors believed that delmopinol might be as effective as chlorhexidine in plaque prevention, where *in vivo* studies would later evaluate this hypothesis (Simonsson, 1991).

In a study evaluating salivary bacterial counts after twice-a-day oral rinses of either delmopinol or chlorhexidine in a panel of 12 males, it was found that 0.2% delmopinol was not as effective as the comparative compound in reducing bacterial counts in saliva, where chlorhexidine reduced counts significantly, while delmopinol led to minimal reduction. As a pioneer study involving delmopinol in 1992, this study did not incorporate factors such as the species of bacteria affected or the reduction of biofilms on tooth enamel (Moran, 1992). Additionally, the collection of post-rinse saliva occurred up to 420 minutes after rinsing, meaning that the initial decline, or potential increase from biofilm dislodgement, was not directly

monitored. Chlorohexidine at the same percentage over a period of six months has also been found to be more effective than delmopinol, however studies have indicated that delmopinol is more tolerable to consumers and less harmful to gums and mouth tissue (Lang, 1998).

Listeria monocytogenes, similar to *S. mutans*, is a gram-positive organism. The effect of delmopinol on gram-positive and gram-negative oral-associated bacteria has been observed by TEM, where 3.2mM (0.1%) and 6.4mM (0.2%) delmopinol hydrochloride concentrations were used with exposure times varying from 1 to 90 minutes. After 1 minute, three of the observed Gram-negative bacteria experienced changes in cell wall components, while the gram-positive bacteria observed showed "little or no" alteration in morphology. This study found that delmopinol binds more to gram-negative rods than gram-positive streptococci (Rundegren, 1995). Despite these findings, it has been previously stated that delmopinol has been shown to be effective for *S. mutans* dislodgement on glass, implying effectiveness for gram-positive *L. monocytogenes*. The observed alteration in gram-negative morphology indicates increased cell binding to delmopinol for gram-negative food pathogens, including *Salmonella enterica* serovars.

As previously mentioned, *S. mutans* is the primary focus of dental studies, as this bacterium is most associated with the dental biofilm/plaque that forms on enamel and threatens its integrity. Other bacteria that may compose an enamel biofilm include other *Streptococcus* spp., *Lactobacillus* spp., and *Actinomyces* spp. In contrast to the three gram-positive genus groups, gram-negative bacteria such as *Veillonella* are also able to grow in oral mucosa, and consequentially in biofilms (Moran, 1992; Rundegren, 1995). While dental studies provide insight regarding how delmopinol might affect non-oral bacteria, there are still many factors that determine how biofilms form within an oral environment, as saliva components and microflora

differ amongst individuals. Overall, previous studies predominantly study the gram-positive *S. mutans* or the total bacterial count or plaque index, meaning that other oral microflora interactions are yet to be researched more specifically. That said, delmopinol hydrochloride has been observed to affect the outer cell membrane more in gram-positive than gram-negative cells (Rundegren, 1995), implying a lessened effect on either prevention or removal, which additionally has been undetermined.

Delmopinol Hydrochloride and Food Pathogens

The Food Science and Technology department of Virginia Polytechnic Institute and State University (Virginia Tech) has thus far been the only university to research the effectiveness of delmopinol hydrochloride on foodborne pathogens. Attached, but non-biofilm cells of *Campylobacter jejuni* have been evaluated on multiple surfaces, including chicken skin, stainless steel and High-Density Polyethylene (HDPE). After spot-inoculation and 10 minutes of cell attachment time, samples were sprayed with 0.5% or 1.0% delmopinol, 0.01% sodium hypochloride for a comparison to a chemical sanitizer or distilled water as a control. Exposure to solutions lasted 1, 10 or 20 minutes before rinsing and enumeration. Findings showed a reduction of 1.26, 3.70 and 3.72 $\log_{10}$ CFU/mL of *C. jejuni* for chicken skin, steel and HDPE, respectively, on attached cells at a concentration of 1.0%. Delmopinol applications resulted in a greater $\log_{10}$ reduction than sodium hypochlorite or distilled water, suggesting beneficial applications in poultry processing environments (Waldron, 2013).

Fresh produce applications have also been evaluated. With the recent threat of foodborne illness related to melons, delmopinol chloride has been explored as a method of reducing biofilms potentially present on melon rinds. Cantaloupe ring plugs previously inoculated with

Salmonella Michigan and allowed to rest for 10 minutes were exposed to delmopinol solutions, where one-hour and 24-hour biofilms were allowed to form thereafter at 37°C. After a sonication step in Butterfield's Phosphate Buffer, enumeration showed a $\log_{10}$ reduction range of 1.6 – 4.4 $\log_{10}$ CFU/mL in comparison to the control for 1.0% delmopinol. This study was able to determine the ability of the compound to dissociate attached bacteria before biofilm formation, where prevention of a biofilm could be observed (Saucedo, 2013). These findings have revealed the promising future of studies incorporating delmopinol in food safety applications.

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CHAPTER III

Listeria monocytogenes and *Salmonella enterica* Biofilm Prevention and Removal on Stainless Steel

Keywords: *Listeria monocytogenes*, *Salmonella enterica*, biofilms, stainless steel, delmopinol hydrochloride

ABSTRACT

Delmopinol hydrochloride, a surfactant used to prevent and remove dental biofilms, was evaluated for paralleled applications in *Listeria monocytogenes* and *Salmonella enterica* Agona biofilms on stainless steel. Stainless steel blanks (#304, 4cm^2, Finish #4) were submerged in a 0.2% or 0.5% delmopinol solution for 1, 5 or 10 minutes before or after biofilm formation. Biofilms were developed on stainless steel blanks by spot-inoculation with 20µL of a 10^9 CFU/mL liquid culture followed by face-down placement on TSA with incubation for 24 hours at 25°C. Pre and post-treatment blanks were individually vortexed in 0.1% peptone water for 90 seconds to dislodge films, and suspensions were serially diluted and plated on TSA followed by incubation at 32°C (*Listeria*) or 37°C (*Salmonella*) for 48 hours. For *L. monocytogenes*, pre-exposure of 0.2% delmopinol provided a slight significant decrease ($P < 0.05$) in bacterial load at all time exposures, while pre-exposure with a 0.5% solution provided no significant effect. All post-exposures lead to a significant decline in biofilm concentrations ($P < 0.0001$). Post-exposures of 10 minutes exhibited a mean $\log_{10}$ reduction of 5.59 and 6.40 $\log_{10}$ for 0.2% and 0.5% delmopinol solutions, respectively. For *S.* Agona, pre-exposure of 0.2% delmopinol provided no significant reduction ($P > 0.05$) while the 10 minute pre-exposure with 0.5% delmopinol exhibited a slight increase in bacterial growth ($P < 0.05$). All post-exposures were effective, where a 10 minute exposure exhibited a mean $\log_{10}$ reduction of 7.65 and 7.75 $\log_{10}$ for 0.2% and 0.5% delmopinol solutions, respectively. Ultimately, the removal effect of delmopinol is significantly greater than a preventative effect for both *L. monocytogenes* and *S.* Agona biofilms on stainless steel.

INTRODUCTION

Listeria monocytogenes is a gram-positive foodborne pathogen with a high mortality rate among immunocompromised individuals and a low infection rate. With a case fatality of 16.25%, pregnant women are likely to experience a stillbirth or miscarriage if infected (Scallan, 2011). Listeriosis outbreaks have traditionally been associated with soft cheeses, ready-to-eat meats and, more recently, produce, where post-process contamination is attributed to the mode of colonization within food (Farber, 1991; McLauchlin, 1987).

The *Salmonella* genus consists of gram-negative bacteria that are aerobic to facultatively anaerobic. There are two species, *S. enterica* and *S. bongori*, where *S. enterica* is the most prevalent species, containing over 2,500 serovars. The bacterial reservoir is often the intestinal tracts of animals, with a large prevalence in poultry, cattle, pigs and sheep (Montville, 2008). Produce-related outbreaks indicate a fecal source of infection, where specific sources may be attributed to agricultural animals that have contaminated water sources. Nontyphoidal *Salmonella* spp. account for the majority of bacterial foodborne illnesses, hospitalizations, and deaths in the United States each year (CDC, 2012), while the number of cases typically increases or remains constant each year (Montville). Outbreaks may be related to greatly varying types of foods, including produce, poultry, eggs and products containing eggs, dairy, spices and peanuts.

Environmentally, *Salmonella* is able to persist for a significant period of time outside of an organism, where survival depends mainly on the availability of water and nutrients. The ability of *S. enterica* to produce biofilms also creates issues regarding environmental stability and growth, as *Salmonella* has been found to persist for at least one year in soil and water (Cavallaro, 2011). Additionally, the ability to survive in dry environments and survive in soil is what has typically lead to fresh produce contamination, where the spread of bacteria can lead to

biofilms on both produce and food-contact surfaces within a manufacturing environment (Cavallaro, 2011).

When essential nutrients and moisture is presented adjacent to a surface, bacteria are able to thrive in high concentration through an initial cell attachment to the available surface. As bacterial concentration rises, cells begin to communicate by means of quorum sensing, where cells emit signals in the form of molecules to other cells in order to invoke a response beneficial to all the bacteria present. Cells begin to exude an extracelluar polysaccharide substance (EPS; also known as exopolysaccharide) to comprise a matrix in which bacteria can embed themselves and form a bacterial community. The ability for communication between cells via Quorum sensing allows for the creation of channels that may carry waste, nutrients and/or water from one grouping of cells to another. The EPS matrix serves as a physical and chemical barrier from detergents, sanitizers and mechanical action, which in turn has a protective effect on the cells. As a biofilm matures and the bacterial concentration is maintained, the microorganism community will expel bacteria into the surrounding environment, causing the spread of contamination in a food manufacturing environment.

L. monocytogenes and *S. enterica* have been known to form biofilms in processing environments on various food-contact surfaces such as stainless steel, polypropylene (among other plastic polymers), glass, and rubber gaskets. *L. monocytogenes* and *S. enterica* have also been found to form biofilms on produce and raw foods, including cantaloupe and chicken skin (Saucedo, 2013; Waldron, 2013). Relative to abiotic surfaces, stainless steel is the most prominent food-contact surface within a processing facility due to corrosion resistance, durability, and general ease of cleaning (Boulange-Peterman, 1996). Biofilms are capable of

development on stainless steel, and further understanding of how biofilms may be prevented and removed on stainless steel is beneficial to the food industry.

Typically detergents with mechanical force and increased sanitizer concentrations have been implemented to remove biofilms and/or cause cell death of biofilm-embed bacteria. However, delmopinol hydrochloride, a compound that has been shown to be effective against dental plaque, may be useful for control of biofilms in the food industry. Delmopinol hydrochloride is a cationic surfactant manufactured initially for dental biofilm applications, where assorted products incorporating the compound at 0.2% are available for consumer-use with the intention of dissociating and preventing enamel biofilms (Sunstar, 2013). Typically, *Streptococcus mutans*, a gram-positive bacterium, has been highlighted in multiple studies with notable success (Rundegren, 1992). Recently, food pathogen applications have been explored (Saucedo, 2013; Waldron, 2013), but studies specific to *L. monocytogenes* biofilms on stainless steel have not been previously investigated. The purpose of this study was to evaluate the effectiveness of delmopinol hydrochloride for the prevention and removal of *L. monocytogenes* and *S. enterica* biofilms on stainless steel.

MATERIALS AND METHODS

Bacterial Strains. *Listeria monocytogenes*, serovar 1/2a, isolate M39503A obtained from the veterinary school of Washington State University was held in 15% glycerol media at -80°C.

Salmonella enterica subspecies *enterica*, serovar Agona was selected based on biofilm-forming capacity and availability from cultures maintained at the Virginia Tech Department of Food Science and Technology.

Culture Preparation. Cultures were prepared by suspending one colony grown on a plate of TSA into 10mL of Trypticase Soy Broth (TSB) followed by incubation at 32°C for *L. monocytogenes* or 37°C for *S.* Agona for 20 to 24 hours. Liquid cultures were transferred to a 15mL conical tube, centrifuged (4000 x g for 10 minutes), where the used media supernatant was discarded and replaced with an equal amount of sterile 0.1% peptone water. The pellet of cells was resuspended in the peptone water by vortexing at 2500RPM. This culture (10^9 CFU/mL) was used as the direct inoculum for stainless steel blanks.

Delmopinol Solution Preparation. Powdered delmopinol hydrochloride (delmopinol; Sinclair Pharmaceuticals Limited; Godalming, Surrey, UK) was diluted in sterile, distilled water. Contents were thoroughly mixed by shaking until the powder completely dissolved. The delmopinol solution was not was not sterilized; therefore, absence of culturable background microflora was confirmed by plating onto TSA. Delmopinol concentrations of 0.2% and 0.5% were prepared in one liter (1L) volumes in screw-cap Erlenmeyer flasks.

Stainless Steel Preparation. Stainless steel, grade 304 (T304), 16 gauge (1.59mm thick) with a #4 finish (satin) (Atlantic Stainless Co., Inc.; North Attleboro, MA) was utilized in this study. or this study, 2cm x 2cm ($4cm^2$) blanks were waterjet-cut to ensure accuracy in sizing.

Stainless steel blanks were cleaned and prepared before biofilm studies occurred. Initially, blanks were placed in acetone for a minimum of 30 minutes. After a rinse with distilled water, blanks were placed in 1N sodium hydroxide (NaOH) for 30 minutes. After the NaOH bath, blanks were rinsed with distilled water before air-drying. This process was adapted from various studies (Breemer, 2001; Chmielewski, 2004; Hood, 1997). Prior to biofilm preparation

and blank inoculation blanks were sterilized with 70% ethanol and allowed to air dry in a biological safety cabinet (Figure A.2).

Biofilm Formation. Stainless steel blanks and media prepared as described previously were inoculated with an overnight culture of *Listeria monocytogenes*, grown in Trypticase Soy Broth (TSB) at 32°C for 20 to 24 hours. After the culture was centrifuged (4000 x g for 10 minutes), supernatant media was discarded and the cell pellet was resuspended in 0.1% peptone water. After vortexing, 20 µL of the 10^9 CFU/mL inoculum was spot-inoculated onto each previously sterilized and dried blank.

After inoculum was added to the finish-side of the blank, a sterile inoculating loop was used to evenly spread the culture across the $4cm^2$ surface. With the use of ethanol-disinfected forceps blanks were transferred finished-side face-down (Figure A.1) onto a TSA plate.

Solid Media Biofilm Development Procedure. After inoculation and face-down placement on TSA, formation of a young biofilm continued over the next 24 hours while incubated at 25°C. After this one-day incubation, all blanks were rinsed with sterile phosphate buffered saline (0.01M, 7.0pH; approximately 15mL per blank) until exhibiting a "clean" appearance (no apparent culture or media present on the surface) before biofilms were dislodged for pre-exposed treatments and controls or treated before dislodgement for post-exposure samples (Figure A.4). The PBS rinsing procedure was consistent for each blank, where a side-to-side stream followed by right angle turn and replicate stream of PBS from sterile squeeze bottle took place for each blank. To rinse the back, there was an additional light stream directed at one side of the four

corners of the blank, allowing for an even flow that gently washed liquid possibly containing planktonic cells from the blank.

Delmopniol Hydrochloride Pre- and Post-Exposure. For the groups exposed to delmopinol before biofilm formation, blanks were placed #4 finish side-up in a 35mm Petri plate containing 5mL of either a 0.2% or 0.5% solutions of delmopinol hydrochloride for 1, 5 or 10 minutes. After delmopinol exposure, 20µL of 10^9 CFU/mL culture was spot-inoculated onto the stainless steel blank before the remainder of biofilm development procedures previously described (Figure A.3).

Treatment groups exposed to delmopinol after biofilm formation were placed #4 finish side-up within a 35mm Petri plate containing 5mL of either a 0.2% or 0.5% solutions of delmopinol hydrochloride for 1, 5 or 10 minutes. After a rinse with an additional light stream of PBS directed at one side of the four corners of the blank, biofilms were dislodged and bacterial counts were enumerated given the procedure explanation followed. Pre-exposed samples and controls were immediately subjected to the dislodgement step after the PBS rinse.

Biofilm Dislodgement from Stainless Steel.

Each rinsed blank was placed into a 50 mL conical tube containing 20 mL 0.1% peptone diluent and vortexed for 90s to dislodge biofilms (Figure A.5). Following this step, a portion of the suspension (1 mL) was used for microbiological analysis.

Control Stainless Steel Blanks. Control stainless steel blanks were inoculated and incubated in the same fashion as other samples except that they were not treated with in the delmopinol

solution. After biofilm development, control blank biofilms were rinsed with PBS, as described above, and immediately dislodged by vortexing.

Enumeration of Bacteria. Tryptic soy agar (TSA) was used for both *L. monocytogenes* and *S.* Agona trials to provide an equivalent set of nutrients and recover both culturable injured and non-injured cells. Plates were incubated for 48 hours at 32°C for *L. monocytogenes* and 37°C for *S.* Agona before counting colonies.

Statistical Analysis. Statistical analysis was conducted using SAS software (SAS Institute Inc.), where the mean in $\log_{10}$ values (relative to CFU/cm^2) were compared for significant difference from the control. The Least Squares Means (LSM) within the GLM statistical output included all three variable levels (concentration [0.2%, 0.5%], order [pre-, post-], and exposure time [1, 5, 10 minutes]) was evaluated to determine significant difference or indifference from both the control and sample means. The alpha-level of significance was set to 0.05 ($\alpha = 0.05$).

RESULTS AND DISCUSSION

Selection of Bacteria Serovars. The *Listeria monocytogenes* strain selected was determined based on biofilm-forming capacity, as well as the "persistence" of the strain ability to be consistently isolated from a dairy processing facility (Borucki, 2003). Given all options, 1/2a serovar, isolate M39503A was selected as a result of exceptional biofilm-forming capacity (Borucki, 2003).

The *Salmonella enterica* subspecies *enterica* serovar selected was determined based on biofilm-forming capacity (Vestby, 2009). Preliminary studies involved *Salmonella* Montevideo

and *Salmonella* Agona, both of which have been shown to form biofilms well. Ultimately, *S.* Agona was chosen in that preliminary biofilms formed exhibited consistently higher CFU/cm^2 than *S.* Montevideo.

Stainless Steel. Grade 304, finish #4 was preferable, as it is the most common stainless steel used for food processing equipment in the United States (Boulange-Peterman, 1996) and is moderately rough, opposed to an overly smooth No. 8 (mirror) finish (Stone, 1985). Between each trial stainless steel blanks were disinfected with ammonium quaternary compounds, rinsed with water and cleaned by placement into an acetone bath followed by a NaOH bath at 30 minutes each solution. NaOH is especially important during this cleansing method given its ability to dissolve biofilm EPS (Parkar, 2004).

Solid Media Biofilm Development Procedure Development. Through multiple preliminary studies this TSA method was designed to allow for biofilm growth on the finished side of the blank only, opposed to submersion biofilm development. Submersion biofilm development involves placing a blank into an inoculated liquid culture so that every facet of the blank is exposed to bacteria, allowing for cell attachment on the surface of the entire blank. This method may lead to discrepancies in CFU/cm^2 calculations, as the rougher sides and the non-finished backside of the blank may form biofilms differently from the finished side. The solid media method allows for only one side of the blank to be accessible for cell attachment, biofilm development and exposure to treatment compounds. These procedures were initially developed for photodynamic treatment studies, as light treatments can only illuminate one surface during treatment, however the beneficial nature of this method lead to its use in this study.

Biofilm Dislodgement Development. After stainless steel blanks were treated and/or rinsed, biofilms were dislodged through the use of vortexing in 50mL conical tubes with 20mL of 0.1% peptone water. Biofilms dislodged from stainless steel through the use of sonication, vortexing or shaking with micro glass beads have been shown to produce statistically insignificant differences with respect to bacterial enumeration after biofilm dislodgement, although scanning electron micrographs (SEM) have shown the effectiveness of removing residual EPS and bacteria on stainless steel surfaces is most effective with microglass beads (Lindsay, 1997). Based on multiple studies, rigorous vortexing for 90 seconds was deemed sufficient for dislodging biofilms prior to enumeration (Breemer, 2001; Chmielewski, 2004; Kryinski, 1992; Lindsay, 1997).

Pre-Exposure to Delmopinol Hydrochloride for *L. monocytogenes* (Figure 3.1). Stainless steel blanks exposed to 0.2% delmopinol for 1, 5 or 10 minutes prior to spot-inoculation and incubation at 25°C for 24 hours exhibited a minimal and insignificant change in comparison to the control blanks. Pre-exposure at this concentration yielded a 0.64, 0.62, and 0.89 $\log_{10}$ reduction in comparison to controls for 1, 5 and 10-minute pre-exposures, respectively. Overall, pre-exposure with a 0.2% delmopinol solution slightly prevented biofilm development.

Stainless steel blanks exposed to 0.5% delmopinol for 1, 5 or 10 minutes prior to spot-inoculation and incubation at 25°C for 24 hours exhibited a minimal and insignificant change in comparison to the control blanks. Pre-exposure at this concentration yielded an increase of 0.054, a decrease of 0.62, and a decrease of 0.89 $\log_{10}$ in comparison to controls for 1, 5, and 10-minute pre-exposures, respectively. Overall, pre-exposure with a 0.5% delmopinol solution did not prevent biofilm development.

Delmopinol-containing products clearly market the use of the compound for biofilm prevention and dissociation. However, in this study pre-exposure of stainless steel blanks to either 0.2% or 0.5% delmopinol resulted in little, if any, notable effect on the concentration of bacteria found within the stainless steel-adhered biofilm. In comparison to the control, all averages in the $\log_{10}$ difference of the CFU/cm^2 enumerated never exceeded 1 $\log_{10}$.

When stainless steel blanks were submerged in a 0.2% delmopinol solution before inoculation and biofilm formation, the apparent bacterial concentration was consistently lower than the control blanks. More specifically, both the individual averages and total average of CFU/cm^2 of samples were lower, with a totaled average range of 0.62 to 0.89 $\log_{10}$ reduction. With these reductions, the difference from the control mean for each time of pre-exposure of 1, 5 and 10 produced a significant difference with an alpha of 0.05 ($P < 0.05$, where $\alpha = 0.05$) With these results, the inference is that 0.2% delmopinol has a slight preventative effect on the formation of *L. monocytogenes* biofilms when grown on stainless steel.

Comparatively, pre-exposure of blanks to a 0.5% delmopinol solution provided minimal change in biofilm production. Sample bacterial concentrations fell within a broader spectrum, where a fraction of samples were found to have had a higher CFU/cm^2 count than the control mean. With these results, the total means for pre-exposed samples were insignificantly different from the control means with p-values of 0.81, 0.87, and 0.27 for 1, 5 and 10 minute exposures, respectively. Additionally, similar to the 0.2% pre-exposed samples, no significant difference was found between pre-exposures for the 0.5% concentration.

It was proposed that the higher concentration of delmopinol would perform better than the lower concentration, however the results imply otherwise. Further study of contributing

factors would be required to understand how a more concentrated solution could have negated the slight effectiveness found at a lower concentration.

Post-Exposure to Delmopinol Hydrochloride for *L. monocytogenes* (Figure 3.1). Stainless steel blanks exposed to 0.2% delmopinol for 1, 5 or 10 minutes after biofilm formation at 25°C for 24 hours exhibited a significant change in CFU/cm^2 in comparison to the control blanks. Post-exposure at this concentration yielded a 3.1, 5.4, and 5.6 $\log_{10}$ reduction in comparison to controls for 1, 5, and 10-minute post-exposures, respectively.

Stainless steel blanks exposed to 0.5% delmopinol for 1, 5 or 10 minutes after biofilm formation at 25°C for 24 hours exhibited a significant change in CFU/cm^2 in comparison to the control blanks. Post-exposure at this concentration yielded a 3.3, 6.5, and 6.4 $\log_{10}$ reduction in comparison to controls for 1, 5, and 10-minute post-exposures, respectively.

In contrast to pre-exposure samples, stainless steel blanks exposed to delmopinol hydrochloride after biofilm development provided a significant decline in bacterial concentration for both concentrations. When submerged within a 0.2% delmopinol solution, the $\log_{10}$ reductions averaged to 3.1, 5.4 and 5.6 $\log_{10}$ for 1, 5 and 10 minute post-exposures, respectively. These $\log_{10}$ reductions were greatly significant to the control means ($P < 0.0001$).

Similarly, the 0.5% solution also exhibited effectiveness in biofilm dissociation. The higher concentration solution yielded a 3.3, 6.5 and 6.4 $\log_{10}$ reduction for 1, 5 and 10 minute post-exposures, respectively. As found with the 0.2% post-exposed samples, these values were significantly different from the controls ($P < 0.0001$). Upon review of Figure 3.1, the 0.5% solution was slightly more effective. Statistically, the 1 minute post-treatment was the same for the 0.2% and 0.5% solutions ($P > 0.05$). With a 5 minute post-exposure, the additional mean

$\log_{10}$ reduction was 1.1 $\log_{10}$ for the higher concentration, while the 10 minute post-exposure led to an additional mean $\log_{10}$ reduction of 0.8. These significantly different results imply that a delmopinol post-exposure of 5 minutes or more is more effective at the higher concentration tested. These results were expected, as the higher concentration was purposed to encourage biofilm dissociation than the lower concentration.

Despite a significant difference between 0.2% and 0.5% solutions at 5 and 10 minute post-exposures, there was no significant difference between 5 and 10 minute exposures within the same concentration group. While a longer time exposure is likely to be more effective, only so much of the biofilm can be dissociated, at which point about half of the total $\log_{10}$ reduction occurs quickly within the first minute and continues within the four minutes thereafter. In short, the effectiveness of delmopinol slightly decreases over time after the initial minute of exposure. Given the similarities between 5 and 10 minute treatments and the vast difference between the 1 and 5 minute treatments for both 0.2% and 0.5% solutions, future studies should be conducted to assess the biofilm dissociation and consequential $\log_{10}$ reduction during the first 5 minutes. Additionally, given the large reduction within the first minute, observing effectiveness in smaller increments could prove insightful.

L. monocytogenes **Comparison of Pre- and Post-Treatments for Both Concentrations (Figure 3.1).** As previously stated, results implying biofilm prevention at 0.2% and not 0.5% are misunderstood. The lower concentration only slightly prevented biofilms at 1, 5 and 10 minutes of pre-exposure ($P < 0.05$), with a slight significant difference, where there was no significant reduction for the 0.5% solution ($P > 0.05$). Given the sharp decline in bacterial concentration

with the post-treatments ($P < 0.0001$), it is quite evident that the post-treatment with delmopinol is highly effective comparatively.

Between the 0.2% and 0.5% post-exposed samples, there was no significant difference between solution concentrations when exposure lasted only 1 minute. Otherwise, there was considerably more dissociation of biofilms using the 0.5% solution with a 5 and 10 minute exposure time. One major similarity was that after 5 minutes of exposure for either 0.2% or 0.5%, there was no significant additional decline, meaning that little dissociation occurred after 5 minutes of contact with delmopinol. Consequentially, the most time-effective measure shown would include a post-exposure of a 0.5% delmopinol solution for 5 minutes.

Between the pre- and post-exposed blanks, results were completely dissimilar, as there is no comparative effectiveness between the amount of time in which the blank was submerged within the solution, regardless of concentration. Regardless of concentration or length of exposure, all post-treatments were significantly more effective in dissociating biofilm-embedded cells than pre-exposures were for preventing biofilms. This implies that delmopinol, at least for *L. monocytogenes*, does not adequately prevent cell attachment, however a minor aversion was observed at the commercial concentration of 0.2%. As a gram-positive microorganism, cell membranes can be altered by delmopinol hydrochloride (Rundegren, 1995), where this factor could have influenced how cell repulsion may have occurred beyond the commonly held positive charge of the compound and the bacteria. Post-treatment of *L. monocytogenes* biofilm-laden stainless steel surfaces is most effective during the initial 5 minutes, apart from the concentrations of interest in this study. Further research could address more specific time checks, as well as the highest concentration in which no further improvement in biofilm removal is observed, as well as how lower or higher concentrations prevent cell attachment.

Pre-Exposure to Delmopinol Hydrochloride for *S.* Agona (Figure 3.2). Stainless steel blanks exposed to 0.2% delmopinol for 1, 5 or 10 minutes prior to spot-inoculation and incubation at 25°C for 24 hours exhibited a completely insignificant change in comparison to the control blanks. Pre-exposure at this concentration yielded an increase of 0.10, a decrease of 0.19, and a decrease of 0.11 $\log_{10}$ in comparison to controls for 1, 5, and 10-minute pre-exposures, respectively. Overall, pre-exposure with a 0.2% delmopinol solution did not prevent biofilm development ($P > 0.05$).

Stainless steel blanks exposed to 0.5% delmopinol for 1, 5, or 10 minutes prior to spot-inoculation and incubation at 25°C for 24 hours exhibited a completely insignificant change in comparison to the control blanks. Pre-exposure at this concentration yielded a decrease of 0.073, an increase of 0.092, and an increase of 0.40 $\log_{10}$ in comparison to controls for 1, 5, and 10-minute pre-exposures, respectively. Overall, pre-exposure with a 0.5% delmopinol solution did not prevent biofilm development ($P > 0.05$).

Delmopinol-containing products clearly market the use of the compound for biofilm prevention and dissociation (Sunstar, 2013). That said, pre-exposure of stainless steel blanks to either 0.2% or 0.5% delmopinol proved no notable decrease on the concentration of bacteria found within the stainless steel-adhered biofilm. In comparison to the control for the 0.2% solution, all pre-exposed samples had a $\log_{10}$ difference in CFU/cm^2 within the same range as the control, with insignificant increases and decreases independent of exposure time ($P > 0.05$).

Comparatively, pre-exposure of blanks to a 0.5% delmopinol solution provided an insignificant change in biofilm production for 1 and 5 minute exposures, where a 10 minute pre-exposure caused a slight and consistent increase in concentration ($P < 0.05$). Overall, there was a

mean increase of 0.4 $\log_{10}$ for the longest treatment before cell attachment and biofilm formation. These results imply that increased treatment time before inoculation, cell attachment and biofilm formation causes an increase in biofilm concentration. This increase, while significant, is still quite minimal.

Most previous studies with delmopinol either monitor *Streptococcus mutans*, a gram-positive organism, or the bacterial count overall, so more insight relative to how gram-negative bacteria interact with delmopinol could be useful (Lang, 1998; Rundegren, 1992). What is known is that cell membranes of gram-positive bacteria are affected more by delmopinol than gram-negative bacteria (Rundegren, 1995), meaning that gram-negative bacteria like *S. enterica* could avoid potentially avoid a repulsion-type interaction.

It was proposed that the higher concentration of delmopinol would perform better than the lower concentration, however the results imply otherwise. Regardless of concentration, biofilm development was not prevented, and in the case of stainless steel blank pre-exposure with the 0.5% delmopinol solution for 10 minutes, there was a minor, yet significant increase in CFU/cm^2 ($P < 0.05$).

Post-Exposure to Delmopinol Hydrochloride for *S.* Agona (Table 3.2). Stainless steel blanks exposed to 0.2% delmopinol for 1, 5 or 10 minutes after biofilm formation at 25°C for 24 hours exhibited a significant change in CFU/cm^2 in comparison to the control blanks. Post-exposure at this concentration yielded a 0.84, 3.1 and 7.7 $\log_{10}$ reduction in comparison to controls for 1, 5 and 10-minute post-exposures, respectively. A large decline took place between 5 and 10 minutes, and all treatments were deemed significantly effective.

Stainless steel blanks exposed to 0.2% delmopinol for 1, 5 or 10 minutes after biofilm formation at 25°C for 24 hours exhibited a significant change in CFU/cm^2 in comparison to the control blanks. Pre-exposure at this concentration yielded a 2.5, 7.6 and 7.8 $\log_{10}$ reduction in comparison to controls for 1, 5 and 10-minute post-exposures, respectively.

In contrast to pre-exposure samples, stainless steel blanks exposed to delmopinol hydrochloride after biofilm development provided a significant decline in bacterial concentration for both concentrations. When submerged within a 0.2% delmopinol solution, the $\log_{10}$ reductions averaged to 0.84, 3.1 and 7.8 $\log_{10}$ for 1, 5 and 10 minute post-exposures, respectively. These $\log_{10}$ reductions were greatly significant in comparison to the control mean $\log_{10}$ in CFU/cm^2 ($P < 0.0001$).

Similarly, the 0.5% solution also exhibited effectiveness in biofilm dissociation. The higher concentration solution yielded a 2.5, 7.6, and 7.8 $\log_{10}$ reduction for 1, 5, and 10 minute post-exposures, respectively. As found with the 0.2% post-exposed samples, these values were significantly different from the controls ($P < 0.0001$). Upon review of the graph it would appear as though the 0.5% solution was more effective for 1 and 5 minute treatments, but just as effective overall. Statistically, the 1 minute post-treatment was approximately three times more effective for the 0.5% solution than the 0.2% solution. With a 5 minute post-exposure with 0.5% delmopinol, the additional mean $\log_{10}$ reduction was 5.1 $\log_{10}$ for the higher concentration, while the 10 minute post-exposure led to an additional mean $\log_{10}$ reduction of 0.2 $\log_{10}$. These results were expected, as the higher concentration was purposed to encourage biofilm dissociation than the lower concentration.

Despite a significant difference between 0.2% and 0.5% solutions at 1 and 5 minute post-exposures, there was no significant difference between the 10 minute exposure between the 0.2%

and 0.5% solutions ($P > 0.05$). While a longer time exposure is likely to be more effective, only so much of the biofilm can be dissociated, at which point about a third of the total $\log_{10}$ reduction occurs quite quickly within the first minute and continues within the four minutes thereafter. Given a full 10 minute post-exposure, both concentrations are equally effective, where the higher concentration is no longer beneficial after 5 minutes of exposure, while the 0.2% solution requires more time to dissociate the biofilm prior to a time period that occurs between 5 and 10 minutes of post-exposure.

***S.* Agona Comparison of Pre- and Post-Treatments for Both Concentrations (Figure 3.2).** With the exception of stainless steel blanks exposed to the 0.5% solution for 10 minutes prior to inoculation and biofilm development, all pre-exposed samples did not prevent cell attachment and growth. Given the drastic decline in bacterial concentration with the post-treatments ($P < 0.0001$), it is quite evident that the post-treatment with delmopinol is highly effective comparatively.

Between the 0.2% and 0.5% post-exposed samples, there was a noticeable difference between solution concentrations when exposure lasted 1 or 5 minutes. There was considerably more dissociation of biofilms using the 0.5% solution within a 1 or 5 minute exposure time, where both concentrations were equally effective after 10 minutes of post-exposure. Consequentially, the most time-effective measure shown would include a post-exposure of a 0.5% delmopinol solution for 5 minutes, where a post-exposure of 10 minutes with a 0.2% solution would be the most economical.

Between the pre- and post-exposed blanks, results were completely dissimilar, as there is no comparative effectiveness between the amount of time in which the blank was submerged

within the solution, regardless of concentration. Regardless of concentration or length of exposure, all post-treatments were significantly more effective in dissociating biofilm-embed cells than pre-exposures were for preventing biofilms. This implies that delmopinol, at least for *S. enterica* Agona, does not prevent cell attachment, where a slight increased attachment might be noted for higher concentrations delmopinol and longer exposure times. Post-treatment of *S.* Agona biofilm-laden stainless steel surfaces is most effective during 10 minutes for the commercial concentration (0.2%) and 5 minutes for the higher concentration of 0.5%. Further research could address more specific time checks, as well as the highest concentration in which no further improvement in biofilm removal is observed, as well as how lower or higher concentrations prevent cell attachment. Additionally, searching for more correlation between pre-treatment and increased cell attachment could be addressed through pre-treatment with higher levels of delmopinol.

Comparison to Dental Studies. Dental applications of delmopinol hydrochloride were primarily studied in the 1990's, where a number of studies evaluated the compound in comparison to chlorhexidine (Moran, 1992). Generally, the overall oral bacterial load is enumerated before and after a single use or over a longer period of time, meaning that the effectiveness relative only to prevention or removal of plaque is not generally studied. This study differs in that stainless steel blanks were separately observed for either biofilm prevention or removal for 24 hours, where a combined prevention and removal effect over a longer period of time was not monitored.

Streptococcus mutans is a gram-positive bacteria highlighted in plaque studies due to its commonality and predominance in oral microflora, where delmopinol has been found to reduce dental plaque formed on glass surfaces (Rundegren, 1992). Akin to a mixture of pre- and post-

treatments, glass slides were subjected to 0.2% delmopinol for two minutes three times throughout the 29 hour biofilm development period. Similar to this study, delmopinol was effective in comparison to the control, where concomitant plaque prevention and removal lost more wet weight after sonication than controls (direct enumeration did not occur). Periodic exposure to delmopinol would similarly reduce foodborne pathogen biofilms, however this study cannot differentiate between the specific prevention and removal action of the surfactant (Rundegren, 1992), however it does support the ability to, at the very least, remove biofilms/plaque from a surface, as seen with all post-treatments for *L. monocytogenes* and *S.* Agona.

Delmopinol that has been radio-labeled before exposure to various gram-negative and gram-positive oral bacteria species, gram-negative bacteria exhibit notable structural changes for both cell wall components and the outer membrane in comparison to gram-positive bacteria, which exhibited no morphological change. While this study did not compare the plaque prevention or removal capability between gram-positive and gram-negative bacteria, it is evident that delmopinol interacts more intensely with bacteria with a gram-negative cell membrane and cell wall structure. The findings that delmopinol bound more readily to gram-negative rods could explain the lack of biofilm prevention after pre-exposures to delmopinol for *S.* Agona, as the amphiphillic nature of delmopinol is more suited for interaction with the compound, where prevention does not occur. The variation in the removal ability between *L. monocytogenes* and *S.* Agona, where $\log_{10}$ reductions were higher for *Salmonella*, could also be the result of this difference in interaction between cells. Additionally, the need for a longer post-exposure time could be the result of a lag phase of effectiveness while the compound binds to the *Salmonella* before biofilm dissociation occurs (Rundegren, 1995).

Other studies have mirrored the removal of biofilms in oral systems, where the overall effect (prevention and removal) has been observed to produce plaque and saliva containing less bacteria overall (Addy, 2006). More immediate observations of bacterial reduction have been noted, similar to this study, where saliva contains less bacteria shortly after exposure to delmopinol (Moran, 1992), similar to the short-term treatments conducted in this study. Studies occurring over a period of six months could more accurately portray daily use of delmopinol in a food manufacturing facility, where preventative and removal effects are compounded, as seen in a study comparing effectiveness of delmopinol among other compounds *in vivo* (Lang, 1998).

While dental studies do not specifically mirror the procedures in this study, the findings reflect the effectiveness of the compound in interacting with biofilms and decreasing the amount of plaque, and therefore the number of bacteria, on a surface. This further supports these findings where 0.2% and 0.5% delmopinol hydrochloride, at least for the post-treatments, acts to remove *L. monocytogenes* and *S.* Agona on stainless steel.

Comparison to Foodborne Pathogen Studies. Two studies have monitored the prevention or removal of biofilms or surface-adhered food pathogen bacteria, where results parallel positive findings in the biofilm reduction of *L. monocytogenes* and *S.* Agona on stainless steel in this study. After inoculation and 10 minutes for attachment for *Camplylobacter jejuni* cells, a 3.70 $\log_{10}$ reduction occurred on stainless steel after exposure to a 1.0% solution for 20 minutes (Waldron, 2013). Additionally, delmopinol has been shown to cause up to a 4.4 $\log_{10}$ reduction for *Salmonella* Michigan after a 1.0% solution exposure on cantaloupe rind surfaces (Saucedo, 2013). These results are similar to the post-treatments in this study, where lower $\log_{10}$ reductions are the result of the *C. jejuni* study incorporating only attached cells and the *Salmonella* study

highlighting a surface much more porous and absorbent than stainless steel. Conclusively, these two studies support the ability for delmopinol to reduce biofilms and consequentially bacterial populations for *L. monocytogenes* and *S.* Agona on stainless steel as found in this study.

CONCLUSIONS

Specific to *Listeria monocytogenes*, it was found that delmopinol hydrochloride provided a slight preventative effect and significant biofilm removal ability. While misunderstood, the 0.2% slightly prevented biofilms from forming to maximum capacity (control level of growth), while the 0.5% concentration provided no beneficial action in prevention. Disregarding the mean value, minimal reduction was evident for some samples, while many samples illustrated a slight increase in growth. All post-treatments were effective ($P < 0.0001$), where the majority of the $\log_{10}$ reduction occurred within the first minute for 0.2% and approximately half of the total $\log_{10}$ reduction occurred within this timeframe for the 0.5% solution. The higher concentration of 0.5% demonstrated better biofilm removal after 5 and 10 minutes of post-exposure, however insignificant reduction occurred after 5 minutes in comparison to the samples post-exposed for 10 minutes ($P > 0.05$). This is similar to the commercial concentration of 0.2%, as insignificant removal also took place between the 5 and 10 minute time checks ($P > 0.05$). Conclusively, a 0.2% delmopinol solution parallels a solution with over twice the concentration (0.5%) within 1 minute of exposure, where a higher concentration of 0.5% is more effective when treatment periods last at least 5 minutes, where exposure time thereafter is minimal.

Specific to *Salmonella* Agona, it was found that delmopinol hydrochloride exhibited an absent preventative effect and significant biofilm removal ability for both 0.2% and 0.5% solutions. All post-treatments were effective ($P < 0.0001$), where the majority of the $\log_{10}$

reduction occurred within the first 5 minutes for the 0.5% solution, while the lower 0.2% concentration required the entirety of the studied timeframe for the majority of the $\log_{10}$ reduction observed. The higher concentration of 0.5% demonstrated over twice as much biofilm removal after 5 minutes of post-exposure in comparison to the 0.2% solution, however insignificant reduction occurred after 5 minutes in comparison to the samples post-exposed for 10 minutes for the 0.5% solution ($P > 0.05$). The commercial concentration of 0.2% gradually exhibited reduction between the 1, 5 and 10 minute time checks, where the majority of removal took place between 5 and 10 minutes of exposure. Relative to both solution concentrations, both were statistically found to be equally effective in biofilm removal after 10 minutes of post-exposure ($P > 0.05$). Conclusively, a 0.2% delmopinol solution parallels a solution with over twice the concentration (0.5%) after 10 minutes of exposure, where a higher concentration of 0.5% is more effective when treatment periods last only 5 minutes, where exposure time thereafter is minimal.

Overall, the preventative effects of the solution proved to be minimal and/or insignificant to the control, where the removal ability of delmopinol hydrochloride was significant for both *L. monocytogenes* and *S.* Agona ($P < 0.0001$). Future studies might address effectiveness over a shorter period of time, solution concentrations between 0.2% and 0.5%, multi-species biofilms, and various food-contact surfaces.

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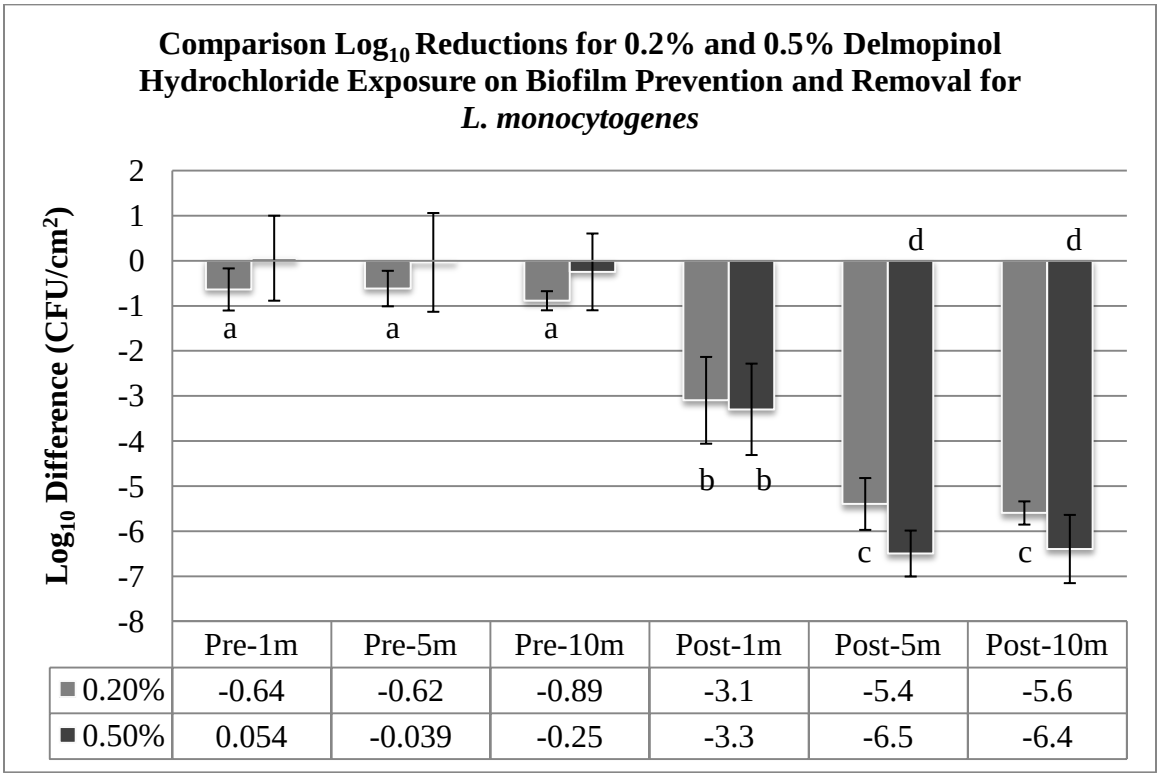
FIGURES

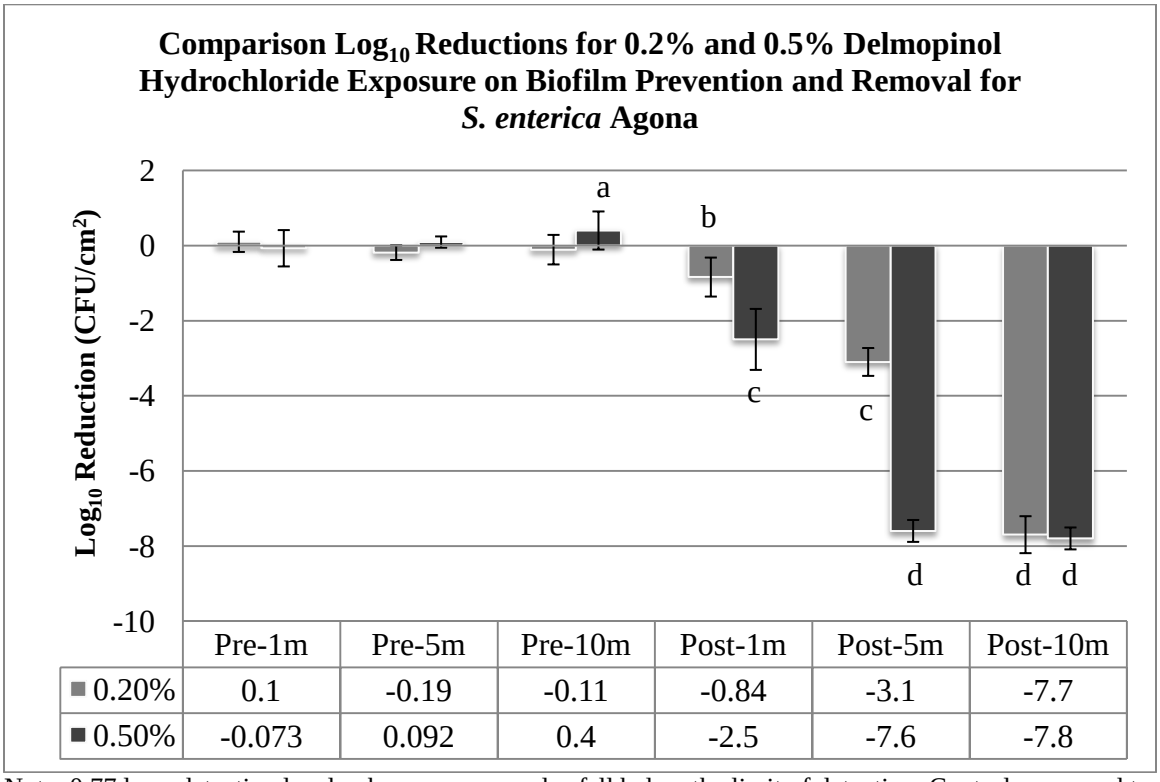
Figure 3.1 Comparison Log_{10} Reductions for 0.2% and 0.5% Delmopinol Hydrochloride Exposure on Biofilm Prevention and Removal for *L. monocytogenes* (n = 84)



	Pre-1m	Pre-5m	Pre-10m	Post-1m	Post-5m	Post-10m
0.20%	-0.64	-0.62	-0.89	-3.1	-5.4	-5.6
0.50%	0.054	-0.039	-0.25	-3.3	-6.5	-6.4

Note: 0.77 log_{10} detection level, where many samples fell below the limit of detection. Controls averaged to 7.22 log_{10} for all trials.

Figure 3.2 Comparison of Log_{10} Reductions for 0.2% and 0.5% Delmopinol Hydrochloride Exposure on Biofilm Prevention and Removal for *S. enterica* Agona (n = 84)



	Pre-1m	Pre-5m	Pre-10m	Post-1m	Post-5m	Post-10m
0.20%	0.1	-0.19	-0.11	-0.84	-3.1	-7.7
0.50%	-0.073	0.092	0.4	-2.5	-7.6	-7.8

Note: 0.77 log_{10} detection level, where many samples fell below the limit of detection. Controls averaged to 7.81 log_{10} for all trials, where all samples within the 'd' group of significance were below the limit of detection (no growth).

APPENDIX

Equipment and Methodology Utilized for Biofilm Formation, Delmopinol Exposure and Biofilm Dislodgement

Figure A.1 Inoculated Stainless Steel Blanks Face-down on TSA

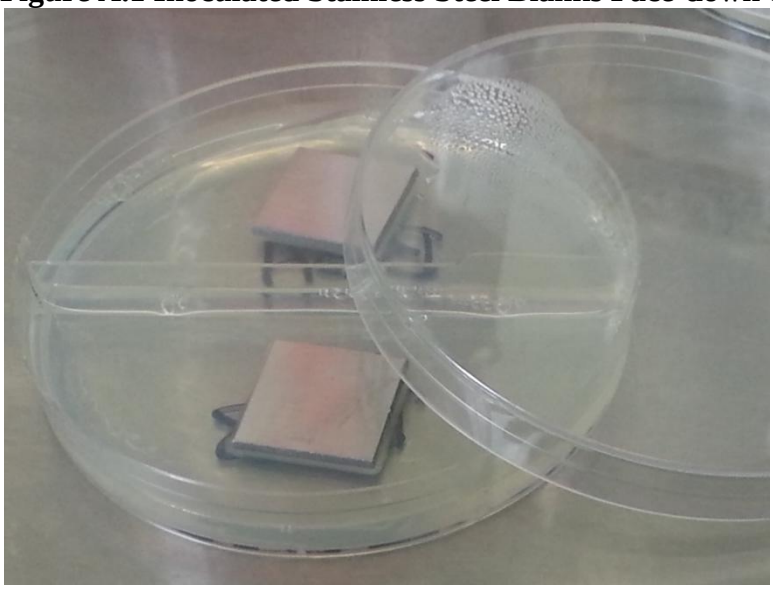


Image by Ellen S. Ewell

Figure A.2 Laminar Flow Hood Set-Up for Inoculation, Treatment and Enumeration

Image by Ellen S. Ewell

Figure A.3 Petri Plates (35mm) with 5mL of Delmopinol Hydrochloride Solution with Submerged Stainless Steel Blanks

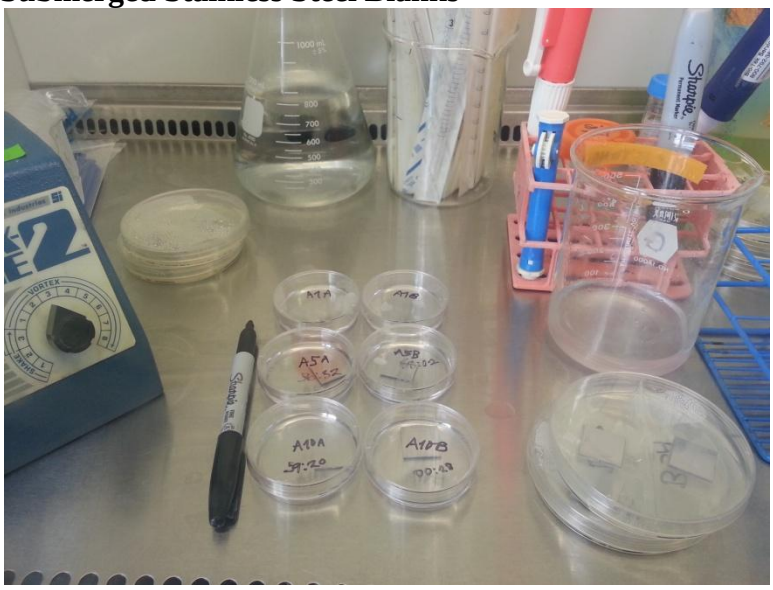


Image by Ellen S. Ewell

Figure A.4 PBS Rinsing Position and Disinfected Forceps Handling of Stainless Steel Blanks Post-Biofilm Formation

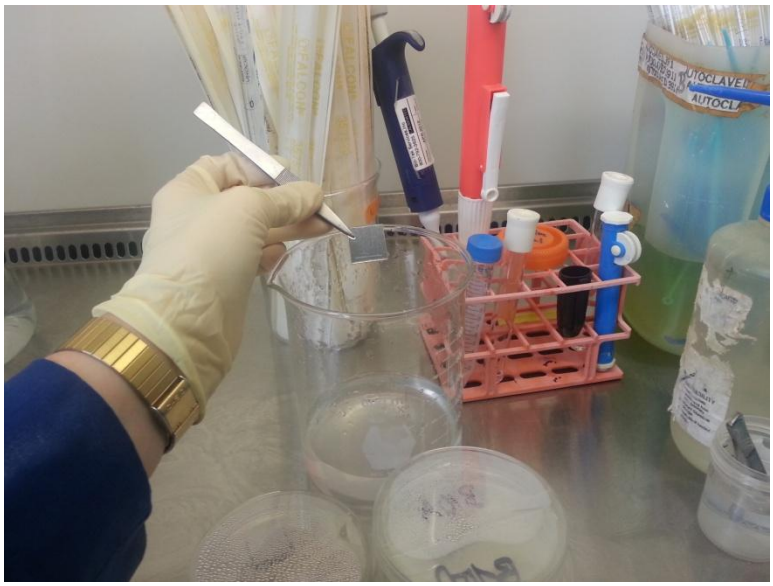


Image by Ellen S. Ewell

Figure A.5 Conical Tubes (50mL) Containing Stainless Steel Blanks and 20mL Peptone Water Prior to Vortexing and Biofilm Dislodgement

Image by Ellen S. Ewell