MICROPLATE LETHALITY ASSAY TO DETERMINE THE EFFICACY OF COMMERCIAL SANITIZERS FOR INACTIVATION OF *LISTERIA MONOCYTOGENES*, *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA* IN BIOFILMS

By

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Bachelor of Science in Biotechnology

Purbanchal University

Biratnagar, Nepal

2014

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December 2017

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ACKNOWLEDGEMENTS

I would like to take this opportunity to express my sincere gratitude towards my advisor Dr. Peter Muriana for continuously motivating and helping me. I really thank him for providing me with such a wonderful opportunity to be a part of his research group and his advice, which helped a lot to complete my research. Secondly, I express my warm thanks to my other committee members, Dr. Ranjith Ramanathan and Dr. Ravirajsinh Jadeja, for their constant support which allowed me to complete my research.

I would also like to acknowledge Dr. Hung King Tiong for constantly motivating me and for providing me with insights into my research. I also thank my lab colleagues Ms. Audrey Boeken and Mr. Dennis Pletcher and the entire faculty and staff of Food and Agricultural Product Center (FAPC) for their support.

Last but not the least, I would like to convey my gratefulness to my family and friends. All the credits of my success till date goes to my parents; Mr. Madhav Prasad Aryal and Mrs. Lila Aryal for their never ending support and prayers. Similarly, special thanks goes to my brother Mr. Dinesh Aryal, sister-in-law Mrs. Megha Baral Aryal and sister Mrs. Kavita Rimal Joshi for their love, encouragement and faith in my ability. The list would not be complete without me thanking my closest friend Ms. Aastha Thapa for always being there to help and support. I would also like to thank all my relatives, Stillwater friends and Nepalese community for their good will.

Name: Manish Aryal

Date of Degree: DECEMBER, 2017

Title of Study: MICROPLATE LETHALITY ASSAY TO DETERMINE THE EFFICACY OF COMMERCIAL SANITIZERS FOR INACTIVATION OF *LISTERIA MONOCYTOGENES*, *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA* IN BIOFILMS

Major Field: MS in Food Science

Abstract: The prevalence of biofilms in food industries has caused serious threats to human health. Different micro-organisms have been found to cross contaminate product itself, equipment and processes in food industries. Though various physical and biological methods have been applied to eliminate biofilms in food industries, chemical methods are still the most common and cost effective ways of biofilm prevention.

The objective of this study was to determine efficacy of commercial sanitizers for inactivation of *L. monocytogenes*, *E.coli* O157:H7 and *Salmonella* in biofilms.

L. monocytogenes, *E.coli* O157:H7 and *Salmonella* spp. were grown in black 96-well microplates and incubated with a fluorescent substrate (5,6-CFDA) to assess the degree of adherence or determine relative fluorescence unit (RFU) values with the help of fluorescent plate reader. Secondly, 7-day old biofilms of the adherent strains were grown in 96-well clear microplates and incubated for an hour at 37º C with different concentrations of enzymes. The recovered cells were then enumerated by plating on TSA plates to evaluate detaching ability of enzymes. Lastly, the 7-day old biofilms were treated with commercial sanitizers at various concentrations for different time periods in 96-well microplates. The reduction in number of cells was quantified by enzymatic detachment and plate counts and qualitatively assessed via scanning electron microscopy (SEM). Repeated Measures (RM) One-Way ANOVA was carried out to see significant differences ($p<0.05$) in the response of different organisms to sanitizer treatment.

L. monocytogenes 99-38, *E.coli* O157:H7 F4546 and *Salmonella* Montevideo FSIS051 were screened as the adherent strains and Trypsin (426.4 U/ml) was determined as a potent enzyme to detach the cells in biofilms. Among the commercial sanitizers, Decon7 (10%) sanitizer mix was found to be the most effective one as just 1 min of treatment with it reduced *L. monocytogenes* 99-38 and *E.coli* O157:H7 F4546 biofilms below limit of detection (2 log CFU/ml) and reduced >7 log CFU/ml of *Salmonella* Montevideo FSIS051 in just 2.5 minutes of treatment.

Thus, the application of new sanitizers (based on combination of several components) followed by enzymatic treatment may be the best option to kill and remove dead biofilms in food processing facilities.

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CHAPTER I

INTRODUCTION

Types of Biofilms

Biofilms are microbial communities that may be found attached to various surfaces. A particularly adherent organism may initiate a biofilm on a surface that becomes the basis for a subsequent biofilm community (Palmer, Flint, & Brooks, 2007). The presence of bacterial cells, a suitable environment (adequate moisture, nutrients, etc.), and an attachment surface is all that is necessary for biofilm formation (Dunne, 2002). Biofilms are involved in many different facets of life.

Environmental. Biofilms are ubiquitous and can be found everywhere from hot springs to frozen glaciers, in streams, on submerged rocks, on plant and animal surfaces or even inside them (Hall-Stoodley, Costerton, & Stoodley, 2004). Some biofilms may even cause disease in animals and crops, while others are important components of the food chain such as biofilms of nitrogen fixing *Rhizobium* on plant roots (Rudrappa, Biedrzycki, & Bais, 2008).

Nautical. Boat hulls are another common niche for biofilm formation. The biofilm

formation further helps in attachment of marine organisms and results in biofouling (Council, 2000). This accumulation or biofouling can slow boat speed, increase fuel consumption, raise maintenance costs and may reduce boat life (Cao, Wang, Chen, & Chen, 2011).

Plumbing. Plumbing involved with showers, sewage pipes, water pipes, cooling, and heating water systems and sinks can have biofilm growth accumulate in them (Hallam, West, Forster, & Simms, 2001; Mahfoud, El Samrani, Mouawad, Hleihel, El Khatib, Lartiges, & Ouaini, 2009). Clogging and corrosion of pipes, unusual or reduced heat transfer in water systems can be the consequences of biofilm formation (Characklis, Nevimons, & Picologlou, 1981).

Medical. Medical devices such as intravenous catheters, cardiac pacemakers, prosthetic heart valves, are also found to be a niche for biofilm formation (Hall-Stoodley, Costerton, & Stoodley, 2004). The presence of biofilms in medical equipment that is inserted into the body may lead to infection or septicemia.

Dental. Biofilms on teeth, such as dental plaques are one of the most common biofilm niches in the human body (Chandki, Banthia, & Banthia, 2011). Dental plaque consists of biofilm with many bacterial and fungal species. The high concentration of bacterial metabolites due to plaque formation may result in dental caries (tooth decay) and gum disease (Marsh, 2006).

Food industries. Biofilm formation is also widely associated with food industries. The processing equipment made up of stainless steel and glasses in food industries have high

surface energy and wettability, which increase the chances of biofilm formation in both food-contact as well as non-contact surfaces (Chmielewski & Frank, 2003).

Bacteria generally attach to surfaces by microbial surface structures (flagella, pilli) and even from electrostatic/hydrophobic properties of surface proteins and molecules (Renner & Weibel, 2011). Once attached, the production of extracellular polysaccharides (EPS) such as carbohydrates, proteins, and nucleic acids, provides the 'glue' that houses the entrapped bacteria. The biofilm matrix may also incorporate clay/slit particles, minerals, crystals, blood components, etc., depending upon the environment they grow on (Donlan, 2002). Microbial groups in biofilms have enhanced persistence, different nutrient utilization patterns, stress response and resistance to antimicrobials compared to planktonic free cells (Kostakioti, Hadjifrangiskou, & Hultgren, 2013).

The main objective of this study was to evaluate different commercial sanitizers against the biofilms of most prominent food borne pathogens namely: *L. monocytogenes*, *E.coli* O157:H7 and *Salmonella* spp. The optimized microplate fluorescence adherence, sanitizer lethality, and enzymatic detachment assays were used to determine the efficacies of the sanitizers.

CHAPTER II

REVIEW OF LITERATURE

Biofilm Structure

Extracellular polymeric substances (EPS). Extracellular polymeric substance (EPS) is a self-produced matrix that holds microbial aggregates together in biofilms (Flemming & Wingender, 2010). In mature biofilms, the EPS matrix may occupy as much as 85% of the volume compared to 15% by bacterial cells (Costerton, Cheng, Geese, Ladd, Nickel, Dasgupta, & Marrie, 1987). It is generally comprised of components such as polysaccharides, proteins, glycoproteins, glycolipids and in some cases extracellular DNA (e-DNA) (Dufour, Leung, & Lévesque, 2010; Flemming, Neu, & Wozniak, 2007). The amount of EPS and its composition may vary between different organisms depending upon the age/maturity and environmental conditions under which the biofilms exist (Mayer, Moritz, Kirschner, Borchard, Maibaum, Wingender, & Flemming, 1999). EPS production is also known to be influenced by the nutrients in the growth medium as excess carbon promotes EPS synthesis while the excess of nitrogen, potassium, and phosphate reduces it (Donlan, 2002; Sutherland, 2001). EPS not only provides shelter to the residing microbial communities but also governs structural and functional aspects of different biofilm communities (Kokare, Chakraborty, Khopade & Mahadik, 2009). Some

of the benefits of EPS include water binding/ preventing desiccation, nutrient diffusion, and preventing disinfectants and antibiotics from reaching to the bacterial cells (Donlan, 2002; Dufour, Leung, & Lévesque, 2010).

Biofilm architecture. The well-known stepwise process for development of biofilms includes i) initial attachment, ii) irreversible attachment, iii) the early development of 3-dimensional biofilm architecture, iv) the maturation of biofilm, and v) dispersion (Stoodley, Sauer, Davies, & Costerton, 2002). The initial or reversible attachment of planktonic cells to surfaces involves hydrophilic/hydrophobic interactions whereas the subsequent irreversible attachment is due to the development of stronger covalent bonds (Chang & Chang, 2002). The attachment process is affected by the physiochemical properties of the surface, hydrodynamics, bacterial properties, and quorum sensing (Kumar & Anand, 1998). After attachment, micro-colonies are rapidly formed and EPS secretion starts, marking the early development of biofilm architecture. Consequently, biofilms begin to mature with higher densities of EPS, channels, and pores, resulting in the redistribution of bacteria away from the substratum (Davies, Parsek, Pearson, Iglewski, Costerton, & Greenberg, 1998). After maturation, microbial cells in biofilms disperse or detach from the aged biofilms in order to survive or search for new niches to colonize. This can occur due to environmental shear forces, fluid dynamics, and abrasion (Kumar & Anand, 1998). Mature biofilms are not a continuous and homogenous monolayer deposits but are a group of micro-colonies heterogeneously embedded in EPS matrix and separated by water channels (interstitial voids) (Donlan, 2002). Besides patchy clumps, biofilms have been found to have various 3-D structures such as pillars or mushroom shapes with water channels in between for the exchange of materials in

and out of the biofilm complex (Schuster & Markx, 2014). Even in mono-species biofilms, there is the presence of phenotypic heterogeneity in different layers (Dufour, Leung, & Lévesque, 2010). The heterogeneity of biofilm architecture is subject to change with the change in internal or external processes (Donlan, 2002). The response of bacteria to the concentration gradients of nutrients, signaling compounds and bacterial waste within mono or multi-species biofilms, results in biological, chemical and structural heterogeneity of the biofilms (Stewart & Franklin, 2008). Similarly, different factors such as adaptation to the local microenvironment, stochastic gene expression, and creation of genetic variants within biofilm result in physiological heterogeneity of biofilms (Stewart & Franklin, 2008).

Physiology of Biofilm Architecture

Nutrient availability. The availability and patterns of nutrient uptake in biofilms differs from that of planktonic cells. The bacterial cells in biofilms exchange nutrients and metabolites by means of water channels between the micro-colonies (Kokare, Chakraborty, Khopade & Mahadik, 2009). The microbes living deep within natural biofilms may often receive low nutrition due to restricted rates of diffusion of nutrients through the biofilm (Petroff, Wu, Liang, Mui, Guerquin-Kern, Vali, Rothman, & Bosak, 2011). Biofilms are also known to provide a suitable environment for syntrophic relationships between the two metabolically distinct bacteria for the exchange of substrates/nutrients (Kokare, Chakraborty, Khopade & Mahadik, 2009).

Genetic transfer. The bacteria in biofilms readily take part in gene transfer or exchange of extra-chromosomal DNA (Donlan, 2002). Horizontal gene transfer is an

important aspect for evolution of microorganisms and has been associated with biofilms in various researches (Madsen, Burmølle, Hansen, & Sørensen, 2012). Hausner & Wuertz (1999) showed that conjugation of bacterial species in biofilm occurs at higher rate than that of their planktonic counterparts. Similarly, the extracellular DNA (e-DNA) available in EPS matrix may increase the competence of biofilm bacteria and hence facilitate transformation or gene transfer (Molin & Tolker-Nielsen, 2003).

Antimicrobial resistance. EPS matrix acts as a diffusion barrier for antimicrobials to invade bacterial cells embedded in a biofilm complex (Donlan, 2002). Even sensitive bacteria with no account for any genetic basis of resistance can have a considerable reduction in antibiotic susceptibility when they reside in biofilm (Stewart & William Costerton, 2001). Research has shown an increase in ampicillin resistance of *Klebsiella pneumoniae* in biofilm compared to its planktonic form (Anderl, Franklin, & Stewart, 2000). A 4-hour old biofilm of *K. pneumoniae* showed 66 % survival against 5000 µg/ml ampicillin compared to complete eradication of free cells. Unlike known mechanisms of resistance in free cells such as efflux pumps, modifying enzymes, and target mutations (Walsh, 2000), there is no rigid evidence to explain resistance by biofilms except perhaps limited diffusion of the inhibitor into the biofilm matrix. Few hypotheses have emerged to explain the mechanism of antimicrobial resistance in biofilms. For example, slow and incomplete penetration of biofilm, presence of resistant phenotype of some bacteria, formation of persister cells and altered environment within biofilms antagonize antibiotic action (Dufour, Leung, & Lévesque, 2010; Stewart & William Costerton, 2001)

Altered stress response. Bacteria in biofilms can express stress-responsive genes in greater amount and switch to forms that are more tolerant. The existence of common regulators, the presence of extracellular polymeric substances, and biofilm heterogeneity are major factors by which biofilms show tolerance against various stressors such as, starvation, heat or cold shock, cell density, pH, and osmolarity (Dufour, Leung, & Lévesque, 2010; Gambino & Cappitelli, 2016).

Quorum sensing. Quorum sensing (QS) is a cell to cell communication mechanism, in which a small diffusible signal molecule is produced, released, sensed and responded to, by bacterial cells (Miller & Bassler, 2001; Cvitkovitch, Li, & Ellen, 2003; Li & Tian, 2012; Waters & Bassler, 2005). Different QS signaling molecules and mechanisms have been explored in various bacterial genera over the years (Irie & Parsek, 2008). When the bacterial population reaches a threshold or quorum level, QS signaling initiates and the auto-inducers bind and trigger functions of target genes (Annous, Fratamico, & Smith, 2009). This ability to communicate helps bacteria to control certain behaviors, such as host colonization, antibiotic production, bioluminescence, sporulation, virulence gene expression, competence, and biofilm formation (Novick & Geisinger, 2008; Rutherford & Bassler, 2012; Williams & Camara, 2009).

Quorum sensing in biofilm formation. Davies, Parsek, Pearson, Iglewski, Costerton, & Greenberg (1998) first described the role of QS in biofilm formation. They discovered the increment in susceptibility of *P. aeruginosa* biofilm towards SDS when the QS system (i.e. las acyl-homoserine lactone) was disabled. Several researchers have subsequently shown the involvement of quorum sensing in multiple stages of biofilm formation (Bai & Rai, 2011; Parsek & Greenberg, 2005). Some of the QS systems seem to promote biofilm

formation whereas some influence maturation and dispersion (Irie & Parsek, 2008). While there is much research indicating biofilm formation as role of quorum sensing, there are others for which the role of QS-regulation in biofilm phenotype variation is ambiguous (Annous, Fratamico, & Smith, 2009). There is still a need for additional research to understand the relationship between quorum sensing and biofilm formation so that we can design strategies to control biofilm formation on food and food processing surfaces (Annous, Fratamico, & Smith, 2009).

Biofilm in Food Industries

The potential of foodborne pathogens and spoilage organisms to form biofilms has raised issues in food industries. A wide range of micro-organisms such as *Listeria monocytogenes, Bacillus cereus, Escherichia coli* O157:H7, *Salmonella* spp., *Pseudomonas* spp., *Staphylococcus aureus*, have been documented to cause biofilm formation on food and food contact surfaces (Dewanti & Wong, 1995; Sharma & Anand, 2002). Apart from bacteria's intrinsic capability to initiate attachment, extrinsic factors such as the food contact surface itself can influence on the level of attachment and ultimately biofilm formation (Srey, Jahid, & Ha, 2013). Food contact surfaces are made up of various materials including stainless steel, glass, polyurethane, teflon, rubber, wood and others (Chia, Goulter, McMeekin, Dykes, & Fegan, 2009; Storgards, Simola, Sjöberg, & Wirtanen, 1999). Sinde and Carballo (2000) found that the degree of attachment and efficacy of sanitizers on surface-biofilms varied considerably between surface types. They found that *Listeria monocytogenes* and *Salmonella* spp. were less adherent to stainless steel compared to rubber and polytetrafluorethylene. However, polytetrafluorethylene was easy to clean and sanitize than the other two. The attachment

of pathogenic bacteria on these surfaces serves as a reservoir of microbial contamination and poses a high risk in the production line (Shi & Zhu, 2009). Different types of food industries or food environment have been linked with different biofilm-forming microbial species. This has raised a great concern for food safety and quality and hence it is indispensable to develop proper cleaning or disinfection procedures for biofilm prevention and control.

Produce industry. With consumers' proclivity towards fresh greens, produce industries have flourished during this trend towards healthy foods. But with the increase in produce intake, there has been a simultaneous and rapid rise in foodborne illnesses associated with fresh produce (Warriner, Huber, Namvar, Fan, & Dunfield, 2009), as well. Some of the microorganisms reported causing produce outbreaks are norovirus, pathogenic *Escherichia coli*, *Salmonella* spp., *Listeria monocytogenes*, *Shigella* spp., *Yersinia enterocolitica*, *Campylobacter spp*., and others (Harris, Farber, Beuchat, Parish, Suslow, Garrett, & Busta, 2003; Ilic, 2011). In 2011, there was an outbreak linked to the whole cantaloupe contaminated with *L. monocytogenes* whose root cause was speculated to be the unsanitary condition of the processing environment. The firm attachment and biofilm formation by *L. monocytogenes* in inaccessible areas and later dispersion during processing, supposedly caused the microorganism contaminate the cantaloupes (Sapers, Miller, Pilizota, & Mattrazzo, 2001). Similarly, various common practices in produce industries including trimming, cutting, slicing, washing, rinsing, and packaging; all of which can serve as primary sources of cross-contamination as a result of biofilm formation and hazardous consequences thereafter (Suslow, Oria, Beuchat, Garrett, Parish, Harris, Farber, & Busta, 2003). The sanitizers normally used in produce industries such

as ozone, chlorine, organic acids, are only effective in reducing 1-2 logs of microorganisms and are usually ineffective against microbial biofilms (Rosenblum, Ge, Bohrerova, Yousef, & Lee, 2012). Areas of concern in produce industries consist the product itself, equipment and process, all vulnerable to biofilm formation and therefore require a rigorous food safety and sanitation plan.

Dairy industry. Dairy industries constituting milk and milk products are highly susceptible to contamination by various microorganisms such as *Enterobacter, Listeria, Micrococcus, Streptococcus, Lactobacillus, Bacillus, Pseudomonas*, and others (Sharma & Anand, 2002; Srey, Jahid, & Ha, 2013). The bacteria present in milk have the ability to attach and aggregate on rubber tubing and stainless steel surfaces. This results in the formation of biofilms in the storage tanks and process lines (Marchand, De Block, De Jonghe, Coorevits, Heyndrickx, & Herman, 2012). The growth of these biofilms in the processing environment increases the risk of microbial contamination of other processed dairy products too. Thus the presence of biofilms containing spoilage and pathogenic microorganisms causes dual risk of product deterioration and disease transmission, which is detrimental to dairy industries (Marchand, De Block, De Jonghe, Coorevits, Heyndrickx, & Herman, 2012).

Fish processing industry. In the fish processing industry, water/ice quality and equipment are major concerns or sources of possible biofilm. *Vibrio spp., L. monocytogenes, Salmonella spp., Bacillus spp., Aeromonas, and Pseudomonas spp.*, are some of the biofilm formers in fish and seafood processing industries (Rajkowski, 2009). Among them, important human pathogen, *L.* monocytogenes, has been reported in crab meat (Brackett & Beuchat, 1990) and fresh water catfish (Jallewar, Kalorey, Kurkure,

Pande, & Barbuddhe, 2007). Similarly, *Pseudomonas spp.* is the predominantly found biofilm former in shrimp industries (Guobjoernsdottir, Einarsson, & Thorkelsson, 2005) as well as in herring, caviar, and cold-smoked salmon plants (Bagge-Ravn, Ng, Hjelm, Christiansen, Johansen, & Gram, 2003). The aquaculture industries such as shrimp hatcheries have storage tanks, polythene pipes, larval tanks, and cement slabs, which are susceptible to biofilm formation (Karunasagar, Otta, & Karunasagar, 1996). The level of biofilm formation can be affected by environmental factors and natural microflora (Shikongo-Nambabi, Kachigunda, & Venter, 2010).

Poultry industry. *Salmonella* spp. and *Campylobacter* spp. are the most commonly found pathogens in poultry and poultry processing areas (Pometto & Demirci, 2015; Rossi, Melo, Mendonça, & Monteiro, 2017). *Campylobacter jejuni* has been found as the persistent microorganism and potential biofilm former in poultry abattoirs (Balogu, Nwaugo, & Onyeagba, 2014; Yang, Jiang, Huang, Zhu, & Yin, 2003). Various studies (Díez-García, Capita, & Alonso-Calleja, 2012; Lamas, Fernandez-No, Miranda, Vazquez, Cepeda, & Franco, 2016; Marin, Hernandiz, & Lainez, 2009) have also observed the ability of *Salmonella enterica* isolated from the poultry industry, to form biofilms. The presence of dust, feces, poultry feed (Marin, Hernandiz, & Lainez, 2009), and transportation of live poultry between production and processing units (Ramesh, Joseph, Carr, Douglass, & Wheaton, 2002) are the major risk factors associated with biofilm formation in the poultry processing industry.

Meat industry. *Salmonella enterica*, *Escherichia coli*, *L. monocytogenes*, and meat spoilage bacteria such as *Pseudomonas* spp., *Lactobacillus* spp., and *Brochothrix thermosphacata* have been recognized as biofilm formers in meat and meat processing

facilities (Giaouris, Heir, Hebraud, Chorianopoulos, Langsrud, Moretro, Habimana, Desvaux, Renier, & Nychas, 2014; Schlegelová, Babák, Holasová, Konstantinová, Necidová, Šišák, Vlková, Roubal, & Jaglic, 2010). The ability of *L. monocytogenes* to grow at low temperature, tendency to adhere on surfaces (Beresford, Andrew, & Shama, 2001; Kushwaha & Muriana, 2009) and resistance to sanitizers (Manios & Skandamis, 2014; Pan, Breidt, & Kathariou, 2006) help them persist in meat processing plants in monospecies or multispecies biofilms (Carpentier & Chassaing, 2004; Fatemi & Frank, 1999). *L. monocytogenes* may be introduced in raw materials (meat) and get associated with walls, drains, slicers, conveyer belts and condensers (Warriner & Namvar, 2009). Destro, de Melo Serrano, & Kabuki (1991) found *L. monocytogenes* in 71.7 % of meat products sampled. Similarly, another study found that *L. monocytogenes* is prevalent throughout processing i.e. in equipment, raw materials and the finished product, during production of fermented sausages (Martin, Garriga, & Aymerich, 2011). Increased prevalence down the process line from slaughter house to cutting or chilling room in pork meat industry (Nesbakken, Kapperud, & Caugant, 1996) have also been observed. The organic residues in meat processing plants and ineffective cleaning procedures, act as suitable factors to facilitate microbial accumulation and biofilm formation (Chmielewski & Frank, 2003). *E.coli* O157:H7 is another pathogen of concern found to form biofilms on various food contact surfaces in meat processing (Dourou, Beauchamp, Yoon, Geornaras, Belk, Smith, Nychas, & Sofos, 2011). The presence of other microorganisms or biofilms on contact surfaces have shown to further enhance the colonization by E. coli (Habimana, Heir, Langsrud, Åsli, & Møretrø, 2010; Marouani-Gadri, Augier, & Carpentier, 2009) in meat industries.

Ready-to-eat (RTE) industry. RTE foods have become popular among people nowadays majorly due to change in lifestyle and desire of convenience. However, RTE foods can be considered as relatively high-risk foods, since the products may be consumed directly without further cooking. The post-cook handling and processing such as weighing, repackaging, loading, etc. are the major reasons of possible cross-contamination with pathogens (Srey, Jahid, & Ha, 2013). Due to the capability of growth at refrigeration temperatures and also formation of biofilms, *L. monocytogenes* is the major concern of RTE food industries (Leong, Alvarez-Ordóñez, & Jordan, 2014). *L. monocytogenes* have been isolated from various RTE foods such as; frozen vegetables, sliced salamis, cream cheese, frozen chicken croquettes, cooked ham, cooked turkey breast and smoked salmon (Di Pinto, Novello, Montemurro, Bonerba, & Tantillo, 2010; Garrido, Vitas, & García-Jalón, 2009). A study (Silagyi, Kim, Lo, & Wei, 2009) has also explored the possible transfer of *E.coli* O157:H7 biofilm from food contact surfaces to RTE deli and produce products.

Elimination of Biofilms in Food Industry

Different strategies have been developed to prevent the formation of, and removal of, biofilms in food industries, such as physical, chemical and biological methods (Kumar & Anand, 1998; Sadekuzzaman, Yang, Mizan, & Ha, 2015). Although physical methods seem to provide gross removal and biological methods such as use of bacteriocin and bacteriophages have been increasing, chemical methods are still the most common and cost-effective method for biofilm prevention (Pometto & Demirci, 2015). The cleaning procedure to eliminate any food debris and residues precedes the application of chemical methods for biofilm removal. Effective cleaning using detergents would help dissolve or

breakdown the EPS matrix and organic material associated with biofilms and help the sanitizers/disinfectants gain access to the exposed bacteria cells (M. Simões, Simões, Machado, Pereira, & Vieira, 2006). The cleaning process can eliminate 90% or more surface-associated microorganisms, but not necessarily kill them. In fact bacteria can redeposit at different locations and form a biofilm if water and nutrients are available (Chmielewski & Frank, 2003). Hence there is a necessity to implement a proper sanitation regimen or chemical treatments (sanitizers/disinfectants/enzymes) in food processing plants to combat the problem of biofilm formation.

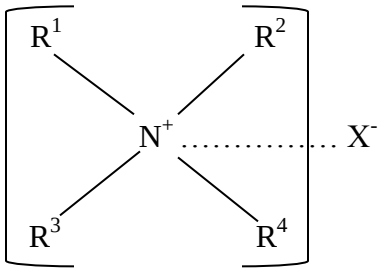
Use of Sanitizers to Eliminate Biofilms

In food industries, the disinfection of surfaces or equipment is mostly done through use of sanitizers (Hood & Zottola, 1995; Karunasagar, Otta, & Karunasagar, 1996; Kumar & Anand, 1998). There are different types of sanitizers which can be grouped broadly as oxidizing agents, surface active compounds, and iodophores (Van Houdt & Michiels, 2010). Widely used sanitizers including halogen-based compounds, peracetic acid (PAA), ozone, and hydrogen peroxide fall under the group of oxidants (Kumar & Anand, 1998). Surface active compounds such as acid anionic compounds and quaternary ammonium compounds (QACs) are also used abundantly in food industries (Van Houdt & Michiels, 2010). However, the thick biofilm matrix comprised of fat, carbohydrates, nucleic acids and protein-based materials, limits the effectiveness of the sanitizers. Moreover, pH, temperature, contact time, water hardness, and concentration are also important factors influencing the effectiveness of disinfectants (Bremer, Monk, & Butler, 2002; Kuda, Yano, & Kuda, 2008). One study showed that *L. monocytogenes* has increased resistance to QACs, chlorine and hydrogen peroxide when the biofilm maturation time was

increased (Pan, Breidt, & Kathariou, 2006). Similarly, some cells might have natural resistance and some might acquire resistance to the sanitizers through genetic exchanges or mutations (Manuel Simões, Simões, & Vieira, 2010). These capabilities possessed by some microbes allow them to grow and persist despite the application of sanitizers. Thus, increased resistance to biocides such as sanitizers, is a concern in food industries and hence the development of new control strategies is highly advocated (Simoes, Bennett, & Rosa, 2009).

Sanitizers for Food Industry

Quaternary ammonium-based compounds (QACs). Quaternary ammonium compounds (QACs) are cationic surface active agents (surfactants) that contain a centrally placed nitrogen atom covalently bonded with four alkyl (R) groups and a negatively charged anion portion (Gerba, 2015).



Basic structure of QACs (Gerba, 2015).

Some of the common examples of QACs are; centrimide, benzalkonium chloride, cetylpyridinium chloride, etc. The activity of QACs is the result of cationic charge that forms electrostatic bonds with negatively charged bacterial proteins (Laopaiboon, Hall, & Smith, 2002; Schmidt, 1997). The antimicrobial activity of QACs primarily involves interaction with membrane proteins, disruption of membrane integrity and progressive

leakage of cytoplasmic contents (McBain, Ledder, Moore, Catrenich, & Gilbert, 2004). QAC is not recommended for use in processing plants that use starter cultures such as plants processing dairy products, cheese, and beer because the residues may inhibit these cultures (Chmielewski & Frank, 2003; Schmidt, 1997). QACs are stable, active, possess low toxicity and have higher efficacy against Gram-positive bacteria, yeasts, molds and lipid containing viruses (Schmidt, 1997). They are however not as effective against Gram-negative bacteria, endospores, and bacteriophages (Ding & Yang, 2013; Gerba, 2015) and has compromised efficacy in the presence of hard water (Schmidt, 1997). The nature and length of alkyl (R) groups determine the antimicrobial activity of QACs with methyl group of 12 to 14 carbon chain showing greater activity (Gerba, 2015). Different studies have suggested variable effects of QACs on different microorganisms. Tapp, Gragg, Brooks, Miller, & Brashears (2013) showed around 3.5 log reductions in both *E.coli* and *Salmonella* population on harvesting knives with use of 200 ppm QAC. Ding & Yang (2013) found that germicidal effect of QAC was enhanced by combined use with alkaline. Furthermore, mixture of QACs and other adjuncts have been used to make antimicrobial products that can target specific organisms (Gerba, 2015). The CFR Title 21 (2015) restricts use of quaternary ammonia compounds to 200 ppm on food contact surfaces.

Chlorine based sanitizers. Chlorine-based solutions are the most common and inexpensive sanitizers used not only in households but also in water systems and food industries and hence the efficacy of other sanitizers is often evaluated by comparison with chlorine-based sanitizers (Park, 2015). The commonly used chlorine compounds include liquid chlorine, hypochlorites, and chloramines (Schmidt, 1997). The most

widely used chlorine disinfectants are hypochlorites which are available as liquid such as sodium hypochlorite (NaOCl; household bleach) or in solid form such as calcium hypochlorite (Rutala, Weber, & Control, 2008). Three different forms: chlorine (Cl_2), hypochlorous acid (HOCl) and monochlorine monoxide (–OCl) may be present in aqueous form depending on the pH (Park, 2015). HOCl is the most active form contributing to germicidal action while –OCl concentration determines cleaning efficiency (Fukuzaki, 2006). Available chlorine (or the amount of HOCl present) depends upon the pH of the solution and also determines the germicidal characteristic of it (Schmidt, 1997). Chlorines are strong oxidizing agents and broad spectrum germicides which have different modes of action of disinfection. They are found to act on microbial membranes, oxidize sulfhydryl enzymes, hinder DNA synthesis and damage DNA, oxidize respiratory components, and inhibit protein synthesis or a combination of multiple factors acting at once (Schmidt, 1997). The mechanism of interaction with proteins involves reaction with amino acid side-chains, cleavage of protein backbones and formation of nitrogen-centered radicals (Hawkins & Davies, 1998). Chlorine dioxide (ClO_2) is another chlorine compound approved by FDA to use in non-food contact surfaces, and poultry and produce process water (Schmidt, 1997). The activity of chlorine is affected by several factors such as pH, temperature, concentration, contact time and organic load (Schmidt, 1997). A study showed that 5 mg/l of sodium hypochlorite (NaOCl) was required to achieve a 5-log reduction of *L. monocytogenes* ribogroup 102–195-S-1 while for same reduction, 50 mg/l of NaOCl was needed against *E. coli* riboroup 102–248-S-4 (Holah, Taylor, Dawson, & Hall, 2002). The presence of organic material or EPS matrix protected cells in biofilms show resistance to chlorine-based compounds.

A research study examined the effect of chlorine on a *Pseudomonas–Klebsiella* mixed biofilm (400 µm thick) and found no penetration of the EPS matrix by 0.062, 0.07, 0.28, and 0.36 mM of chlorine concentrations in bulk liquid even after 1-hour of exposure (De Beer, Srinivasan, & Stewart, 1994). However, some studies such as one by (Toté, Horemans, Berghe, Maes, & Cos, 2010) suggested sodium hypochlorite as one of the potent antimicrobial against *Staphylococcus aureus* biofilms. They found that 1 % NaOCl is able to significantly reduce (almost complete eradication) *S. aureus* in biofilms within 1 minute of contact. Chlorine-based sanitizers might have some disadvantages or limitations associated with their use. One of the major disadvantages is corrosiveness to metal surfaces as observed by (Laycock, Stewart, & Newman, 1997) in passive corrosion of stainless steel. Thus the code of federal regulations (CFR) limits the concentration of sodium hypochlorite on food contact surfaces to no more than 200ppm (FDA, 2014). The stability of sodium hypochlorite may be affected by various factors such as pH, temperature, and exposure to UV light, and series of decomposition reactions might take place (Park, 2015). These decompositions degrade the HOCl and –OCl components and hence reducing the bactericidal and cleaning effectiveness of chlorine based compounds.

$2NaOC1 \longrightarrow NaClO_2 + NaCl$

$NaOCl + NaClO_2 \longrightarrow NaClO_3 + NaCl$

$-OCI + 2HOC1 \longrightarrow C1O_3^- + 2HCl$

$2OCl- \longrightarrow 2Cl^- + O_2$

Hydrogen peroxide. Hydrogen peroxide falls under inorganic group of peroxy compounds unlike PAA which is grouped under organic peroxides (Schmidt, 1997).

H_2O_2 is clear, colorless liquid and environment friendly (non-toxic) sanitizer widely used in medical field and also in food industries (McDonnell & Russell, 1999). Hydrogen peroxide is effective against a broad spectrum of microorganisms including viruses, bacteria, bacterial endospores and yeasts (Chmielewski & Frank, 2003; McDonnell & Russell, 1999). The primary mode of action of H_2O_2 is through oxidization and production of hydroxyl (•OH) free radicals (McDonnell & Russell, 1999). These free radicals can attack and disrupt membrane lipids, target DNA and proteins (sulfhydryl bonds) and affect other essential cellular components (Rutala, Weber, & Control, 2008). Studies on the efficacy of H_2O_2 have shown it as a potent disinfectant in food industries. (Toté, Horemans, Berghe, Maes, & Cos, 2010) showed hydrogen peroxide being active against both biofilms and viable masses of *S. aureus* and *P. aeruginosa*. Similarly, a standard H_2O_2 solution of 4% w/v was found to considerably reduce populations over 8 log with minimum inhibitory concentration of 0.125 to 0.25% for *B. subtilis*, 0.0625 to 0.0938 % for *S. aureus* and 0.125 to 0.376 % for *E. coli* (Penna, Mazzola, & Silva Martins, 2001). Hydrogen peroxide is extensively used in produce industries to sanitize surfaces (Ukuku, 2004) of whole and fresh cut melons, showing effective reduction of microbial populations. The advisable concentration of hydrogen peroxide to be used in food contact surface is 550-1100 ppm (FDA, 2014).

Peroxyacetic acid. Peroxyacetic acid (PAA) is also simply known as peracetic acid. It is a stronger oxidizing agent than chlorine and has molecular formula $C_2H_4O_3$. Commercially available PAA is mainly in the equilibrium form of a quaternary mixture of acetic acid, hydrogen peroxide, PAA, and water as shown by the following chemical equation (Kitis, 2004):

$H_2O_2 + CH_3CO_2H \rightleftharpoons CH_3CO_3H + H_2O$

H_2O_2 = hydrogen peroxide; CH_3CO_2H= Acetic acid and CH_3CO_3H= Paracetic acid.

The mixture has an acrid odor and a low pH (2.8) and is usually manufactured in concentrations of 5 to 15% (Srey, Jahid, & Ha, 2013). It can also be produced by an ozone catalyzed autoxidation of acetaldehyde. The popularity of PAA as a sanitizer is due to various advantages. Some of them are scope of action against different microorganisms including bacteria, yeast, fungi, the ability to enhance the removal of organic material and endotoxins, decomposition into harmless byproducts, and the absence of toxic residues (Rutala, Weber, & Control, 2008). Similarly, PAA is not deactivated by enzymes such as catalase and peroxidase and can be applied over a wide range of temperature (0-40∘C) and pH (3-7.5) (Ding & Yang, 2013). However, peracetic acid can show corrosive responses against materials such as, brass, copper, plain steel, and galvanized iron but modifications in pH and the use of additives can counter corrosion (Kitis, 2004). The CFR Title 21 prohibits use of peracetic acid above 200 ppm for food contact surfaces (FDA 2014). It is considerably unstable with uncontrollable decomposition rates and the rate of decomposition is greater when it's diluted (Asensio, Sanagustin, Nerin, & Rosero-Moreano, 2015). The mode of action of PAA like any other oxidizing agent is denaturing proteins, dislocating or rupturing the cell wall, and oxidizing sulfhydryls and sulfur bonds in enzymes and other metabolites (Rutala, Weber, & Control, 2008). Different researchers have been conducted to demonstrate the efficacy of PAA as sanitizer against biofilms. (Fatemi & Frank, 1999) found that 80 mg/l of PAA is more effective than same concentration of chlorine to inactivate *Listeria/ Pseudomonas* biofilm on stainless steel surface with milk soil. Similarly, peracetic acid has been found

to eliminate viable *S. aureus* (reduction by 98%) and *P. aeruginosa* (99% reduction) on surfaces with only 1 min of contact time but not effective against the same bacteria in biofilm matrix (Toté, Horemans, Berghe, Maes, & Cos, 2010).

Enzymatic Detachment of Biofilm Cells

The dead biofilms left behind by bactericidal sanitizers may facilitate the reattachment of cells and new biofilm formation. To prevent that, enzymes treatment may be used for detachment and removal of biofilm and hence elimination of biofilms. Enzymes such as glycosidases, proteases, and deoxy-ribonucleases have been found to degrade the extracellular polymeric matrix and disperse cells in mature biofilms (Kaplan, 2010). The specific enzymes required to remove biofilms may vary with the type of microflora or matrix embedded in the biofilms (Lequette, Boels, Clarisse, & Faille, 2010) and the heterogeneity of EPS matrix (Kumar & Anand, 1998; Srey, Jahid, & Ha, 2013). For example, protease enzymes worked better than amylases to degrade EPS of *Pseudomonas fluorescence* (Molobele, Cloete, & Beukes, 2010). Similarly, combinations of various enzymes, most often proteases and polysaccharide hydrolyzing enzymes have been proved effective to breakdown the EPS constituents (Meyer, 2003). Apart from enzyme-enzyme combinations, synergistic use of enzyme with surfactants has also shown considerable increase in disinfection efficacy (Jacquelin, Le Magrex, Brisset, Carquin, Berthet, & Choisy, 1994). Wang, Wang, Xing, Wu, Xu, & Zhou (2016) showed that CTAB combined with cellulase eliminated mature biofilm of *Salmonella* in meat processing environments. In the same way, solubilizing enzymes in buffer containing surfactants, chelating agents, was found to enhance biofilm removal (Lequette, Boels, Clarisse, & Faille, 2010). Research has established that enzymes are non-toxic and are

good environmentally friendly alternatives for biofilm removal (Srey, Jahid, & Ha, 2013). However, enzyme specificity, the high cost, and low commercial accessibility of enzymes, has limited the use of this method (Manuel Simões, Simões, & Vieira, 2010).

CHAPTER III

MATERIALS AND METHODS

Bacterial Cultures and Growth Conditions

Bacterial cultures. Bacterial cultures used in this study are listed in Table 1. Most of these strains were used for initial adherence screening assay to confirm or identify high level adherence. The selected adherent strains were then further used for sanitizer application and enzyme detachment assays.

Growth conditions. Cultures were stored frozen by centrifuging 9 ml of overnight cultures and re-suspending the pellets in 2-3 ml of fresh sterile BHI broth containing 10 % glycerol and then stored in glass vials in an ultra-low freezer (-80°C). The frozen stocks were thawed and revived by transferring 100 µl into 9 ml of Brain Heart Infusion (BHI) broth. The BHI tubes with cultures were then incubated overnight at 30º C and sub-cultured at least twice before use in assays. Microbial enumeration for all the assays was carried out on Tryptic Soy Agar (TSA) plates, plated in duplicate.

Table 1. Bacterial cultures used in this study

Bacterial culture	Source	Reference
Listeria monocytogenes CW35	Retail RTE frankfurters	Wang & Muriana, 1994; Tiong & Muriana, 2016
Listeria monocytogenes 99-38	Retail ground beef	Gamble & Muriana, 2007; Kushwaha & Muriana, 2009; Tiong & Muriana, 2016
Escherichia coli 0157:H7 2-RR_2	Muriana culture collection	P. Muriana
Escherichia coli 0157:H7 2-ML_2	Muriana culture collection	P. Muriana
Escherichia coli 0157:H7 ATCC 43888	Muriana culture collection	P. Muriana
Escherichia coli 0157:H7 F-4546	Muriana culture collection	P. Muriana
Salmonella Typhimurium NAL100	Muriana culture collection	P. Muriana
Salmonella Enteritidis 13076	Muriana culture collection	P. Muriana
Salmonella Enteritidis E1-32 CDC	Muriana culture collection	P. Muriana
Salmonella Enteritidis E1-40 CDC	Muriana culture collection	P. Muriana
Salmonella Heidelberg 8326	Muriana culture collection	P. Muriana
Salmonella Heidelberg F5038BG1	Muriana culture collection	P. Muriana
Salmonella Senftenburg 43845	Muriana culture collection	P. Muriana
Salmonella Hadar MF 60404	Muriana culture collection	P. Muriana
Salmonella Montevideo FSIS051	Muriana culture collection	P. Muriana
Salmonella enterica ser. Thompson	Muriana culture collection	P. Muriana
Salmonella Senftenburg 43846	Muriana culture collection	P. Muriana

Fluorescent Microplate Biofilm Adherence Assay Optimization

Various parameters were tested on biofilms grown in microplates such as use of fluorescent dyes, number of wash times, and age of biofilms before continuing with specific assays.

Fluorescent substrate. The mixed-isomer substrates 5,6-carboxyfluorescein diacetate (5,6-CFDA) and 5,6-carboxyfluorescein diacetate, succinimidyl ester (5,6-CFDA, SE; Molecular Probes/Invitrogen, Carlsbad, CA) were compared for the ability to produce fluorescence signals in a microplate biofilm assay and hence to determine which one was a more suitable substrate for fluorescence assay. The 5, 6- CFDA was dissolved in dimethyl sulfoxide (DMSO) to get 2% (w/v) stock solutions. Working solutions were prepared thereafter by allocating 10 µl of the stock solutions to 1ml of Tris buffer (0.05 M, pH 7.4).

Microplates. We used black, non-treated 96-well flat-bottomed microplates (Cat: 237105, NUNC, Denmark) (Fig.1) to perform the fluorescence assay and determine adherence of bacteria. Black plates prevent "cross-talk" from neighboring wells during fluorescent measurement and fluorescence signals can be read from top. When fluorescence was not needed, a different set of sterile Falcon 96-well clear, non-treated flat-bottomed polystyrene microplates (Cat: 351172, Corning, NY) (Fig.2) were used to grow microbial biofilms and perform subsequent washing, lethality, and detachment/enumeration assays.

Figure 1. A black 96-well microplate (NUNC).

Figure 2. Clear, flat-bottomed microplate (Falcon).

Microplate washer. The microplates used for detachment and lethality assays were subjected to a wash treatment in a Biotek Elx405 Magna plate washer (Ipswich, Suffolk, United Kingdom) (Fig. 3). This microplate washer was connected to separate wash-liquid containers (10% bleach, de-ionized water and Tris buffer) as well as waste containers. The plate washer has 96 pairs of needles (one for aspiration; another for dispensing) to

draw liquids into, and out of, the wells as well as a shake parameter (to shake the plate to re-suspend settled cells before washing).

Figure 3. Biotech Elx405 Magna plate washer.

Washing procedure. The microplates used in this study were subjected to different sets of rinsing and washing procedures in a plate washer in order to wash off loose attached cells. Different settings can be used to clean the needles, dispense wash solutions, rinse the wells and wash off the planktonic cells in the wells of microplates. Before washing the 96-well microplates, maintenance cycles were performed in the plate washer and the needles were rinsed first by 10% bleach (2 times) followed by de-ionized water (3 times)

and Tris buffer (2 times) to remove any deposits or contaminants. After the rinses, microplates were washed with Tris buffer for various numbers of times (3 times, 9 times, and 15 times) using the shake option in Magna Plate Washer (Fig. 3) so that optimal wash time could be determined and used in subsequent assays.

Enzymatic Detachment/ Enumeration Assay

In this research we investigated the use of enzymes such as pronase E, trypsin, bax protease, papain, cellulose, and lipase to screen for their ability to detach and remove bacteria entrapped in biofilms.

Pronase E. Pronase E (Cat: P5147, Sigma-Aldrich, St. Louis, MO) from *Streptomyces griseus* was obtained in powdered form with activity of 5.3 U/mg. The working solution was prepared at concentration of 500 U/ml. For this purpose, 0.4715 gm of Pronase E powder was added to 5 ml of sterile Tris (0.05 M, pH 7.4), dissolved and filter sterilized to get a stock solution.

Trypsin. Trypsin (Cat: T4549, Sigma-Aldrich) from porcine pancreas was another protease enzyme used in this research. It was obtained in liquid (solution) form with an activity of 1485.9 U/ml. For our research purpose, we used trypsin in two different concentrations, the original (1485.9 U/ml) and diluted to 426.4 U/ml.

Bax protease. Bax protease (DuPont Qualicon, Wilmington, DE) was obtained as a solution and used as per manufacturer's guideline [12.5 µl in 1ml Tris (0.05M, pH 7.4)]

(Gamble & Muriana, 2007). The specific protease is undisclosed so we used it at the working strength recommended by the manufacturer.

Papain. Papain, *Carica papaya* (Cat: 5125, EMD Millipore Corp., Billerica, MA) had listed activity of 31850 U/mg and stock solution was prepared by adding 0.31 gram in 10 ml Tris (0.05 M, pH 7.4) i.e. concentration of 1000 KU/ml.

Cellulase. Cellulase from *Aspergillus niger* (Cat: C1184, Sigma-Aldrich) was used in same concentration as pronase E i.e. 100 enzyme units (U) per 200 µl. The activity for cellulase was marked as 1.3 units/ mg solid and hence 1.92 grams of cellulase powder was added to 5 ml Tris (0.05 M, pH 7.4) to get desired stock solution concentration of 500 U/ml.

Lipase. Lipase from *Candida rugosa* (Cat: L1754, Sigma-Aldrich) had activity of 1170 U/mg solid. Lipase powder weighing 2.14 mg was dissolved in 5 ml of Tris (0.05 M, pH 7.4) to get a concentration of 500 U/ml. It was then filter sterilized before use.

The mass (weight) of powdered enzymes needed to get the desired stock solution concentration in U/ml, were calculated using following formula;

Activity of enzyme per mass of material = (A) U/mg

Total stock volume made = (V) ml

Desired final concentration of enzyme solution= (C) U/ml

$$\text{Mass of solute to be dissolved (m)} = \frac{(C) \times (V)}{(A)} \text{ mg}$$

Enzymatic detachment/enumeration. In order to obtain a plate count enumeration of biofilm bacteria, either before (controls) or after sanitizer treatment (experimental), we had to evaluate and optimize the best method to detach and recover viable cells. For this purpose, overnight cultures (10^9 CFU/ml) of three strongly-adherent pathogenic microbes: *Listeria monocytogenes* 99-38, *E. coli* O157:H7 F-4546, and *Salmonella* Montevideo FSIS051 were diluted 5-fold to 10^4 CFU/ml in BHI broth. A 200 µl aliquot of each culture was allocated in triplicates into Falcon 96-well microplates. The microplates were then incubated at 30°C for 24 hours. After 24 hours, the wells were washed 3 times with Tris buffer (0.05 M; pH 7.4) in a Biotec Elx405 Magna plate washer. A 'shaking' option was used to wash off loosely adherent cells in addition to resuspending settled planktonic cells. This was followed by the addition of fresh BHI (200 µl) into the wells and an additional incubation for 24 hours at 30°C. The same process of washing with Tris buffer and adding fresh BHI into wells was repeated each day for one week. After 7 days of washing and incubating, the final wash with Tris buffer using the plate washer (with shaking) was performed and 200 µl of different enzymes at the earlier stated concentrations were transferred into the experimental wells. For controls, only Tris was poured into the wells with bacterial biofilms. After the addition of enzymes, the microplate was incubated for an hour at 37°C. Finally, to get detached cell counts, the solution in test wells were plated on Tryptic Soy Agar (TSA) plates and incubated at 30°C for 24 hours.

Pathogen biofilm screening/ confirmation

***Listeria monocytogenes*.** A convenient fluorescence assay (Gamble & Muriana, 2007) was used to screen and identify adherent characteristics of *L. monocytogenes*. For this

purpose, overnight cultures of different strains of *L. monocytogenes* were diluted from 10^9 CFU/ml to 10^4 CFU/ml in BHI broth. 200 µl of each culture was then allocated in triplicates into the black 96-well microplate (NUNC). The microplate was then sealed with parafilm and incubated at 30°C for 24 hours. After incubation, for removal of free-floating/planktonic cells from the wells, the plate was washed 3 times (with the shaking option) with Tris buffer (0.05 M; pH 7.4) in a Biotec Elx405 Magna plate washer. This was followed by addition of fresh BHI (200 µl) into the experimental wells and additional incubation for 24 hours at 30°C. Again the three times wash with Tris buffer using the plate washer was performed. After the wash, 200 µl of light sensitive 5,6-carboxyfluorescein diacetate (5,6-CFDA) dye was added to the experimental wells and incubated for short period of 15 min at 37°C. After incubation with the fluorescent substrate solution, the microplate was washed three times again with Tris buffer (0.05 M; pH 7.4) and the wells were replaced by 200 µl of fresh Tris buffer. The Tecan Genios fluorescent plate reader (Phenix Research products, Hayward, CA) and associated Magellan software was used to measure the degree of fluorescence from each of the wells. For the fluorescence reading, a fixed signal gain of 75% with excitation at 438 nm and emission at 535 nm was used (Gamble & Muriana, 2007). The attachment of cells was further confirmed by detaching with protease enzyme and plating viable counts.

***E.coli* O157:H7.** Four strains of *E. coli* O157:H7: $2RR_2$, $2ML_2$, ATCC 43888 and F4546 were also screened together with *L. monocytogenes* strains in microplates. The same method was followed for *E.coli* O157:H7 where the strains were diluted 5-fold (from 10^9 CFU/ml to 10^4 CFU/ml), allocated into microplate wells (200 µl in each well) and incubated for 24 hours at 30°C. After changing fresh BHI media in wells and incubating

for an extra day, the microplates were then washed with Tris buffer and again, incubated with fluorescent dye substrate (5,6-CFDA) (the same as with *Listeria*). The relative fluorescence units (RFU) of the test strains were observed by use of plate reader in fluorescence mode. The blank wells were filled with Tris and taken as negative control for this assay. For confirmation of levels of attached cells, enzyme detachment and plate count enumeration of the same wells was also performed.

***Salmonella* spp.** The same microplate adherence assay was also used to screen 10 different strains of *Salmonella* spp. for their adherence characteristics. As with *L. monocytogenes* and *E.coli* O157:H7, the cultures were grown in microplates and fluorescence of attached cells of the strains was quantified using the plate reader after 48 hours of attachment (as described above). For this assay, strongly adherent strains of *L. monocytogenes* and *E.coli* O157:H7 were used as positive controls and blank wells as negative control. The enzymatic detachment of test wells was done to confirm the presence and numbers of attached cells of *Salmonella* strains.

Sanitizer biofilm microplate assay

Biofilm lethality protocol. Biofilm lethality assays using various sanitizers were carried out in 96-well microplates. The three strongly adherent strains, *Listeria monocytogenes* 99-38, *E. coli* O157:H7 F-4546 and *Salmonella* Montevideo FSIS051 were used to form 7 day old mature biofilms (media replaced daily). The 7 day biofilms were washed three times with Tris buffer (0.05M, pH 7.4) in the plate washer (with shaking) and 200 µl of different concentrations of various sanitizers were added thereafter. After incubating

sanitizers for the various assigned treatment periods, the treated microplates and control (with buffer treated) were again washed with Tris, aspirated and then 200 µl Dey-Engley (DE) neutralizing buffer (Hardy Diagnostics, Santa Maria, CA) was added to the wells. The microplates were left for 5 minutes to neutralize the effects of sanitizers.

Sanitizer treatment of microplate biofilms.

Different sanitizers such as Bi-Quat (Birko), 10-chlor (Birko), Sterilex (Sterilex Corporation), KC-610 (Packers Chemicals), and D7 (Decon 7) were used in this study to analyze their effects on biofilms.

Bi-Quat. Bi-Quat (Birko, Henderson, CO) was used in the concentration of 200 ppm (i.e. 0.08 gallons per 40 gallons of water or 2 milliliters in 1 litre of water). The effects of 200 ppm bi-quat on pathogenic biofilms were observed over different time periods; 15 minutes, 30 minutes, 1 hour, and 2 hours.

10-Chlor. A 10-Chlor (Birko, Henderson, CO) (10% sodium hypochlorite) was used in two different concentrations of 200 ppm (2.5 fl. oz. of the product per 10 gallons of water) and 1000 ppm (12.5 fl. oz. of the product per 10 gallons of water). The biofilms were separately incubated with 200 ppm and 1000 ppm of 10-chlor for four different time periods; 5 minutes, 15 minutes, 30 minutes, and 1 hour.

Sterilex solution. Sterilex solution (Sterilex Corporation, Cockeysville, MD) is a two part liquid concentrate mixed together at the time of use. The two different parts are: Part1 (Ultra Disinfectant Cleaner Solution 1) and Part 2 (Ultra Activator Solution). Two different concentrations of working sanitizer solution were made to test efficacy of this product. Part 1 (Ultra Disinfectant Cleaner Solution 1) and Part 2 (Ultra Activator

Solution) solutions were separately mixed with 10 ml and 20 ml of water to get two different concentrations of 10% and 5 % respectively. The biofilm treatment time periods were 1 minute, 2.5 minutes, 5 minutes, 10 minutes and 20 minutes for 10 % and 2.5 minutes, 5 minutes, 10 minutes and 20 minutes for 20 ml mix.

Decon7 solution. Decon7 solution (Decon ™ Seven Systems, Scottsdale, AZ) came in three parts: Part 1: A surfactant (quaternary ammonium compound) Part 2: An oxidizer (hydrogen peroxide) and Part 3: An optional accelerator (diacetin). These three parts were mixed in the ratio 2:2:1 and used in two different concentrations of 5% and 10% to assess efficacy against biofilms. 0.4 ml each of Part 1 and Part 2 and 0.2 ml of Part 3 solutions (i.e. 2:2:1 mix) were added to two different volumes; 10 ml and 20 ml of water. This gave us the 5% and 10 % of solution that we wanted to test the biofilms with. Similar to sterilex solutions, 5% decon mix had treatment time periods of 2.5 minutes, 5 minutes, 10 minutes, and 20 minutes and 10 % was treated for 1 minute, 2.5 minutes, 5 minutes, 10 minutes, and 20 minutes.

KC-610. KC-610 (Packers Chemical, Kieler, WI) is a peroxyacetic acid (PAA) based antimicrobial solution which was used as per manufacturer's instructions at concentration of 6.1 oz per 6.0 gal of water. The active ingredients of the solution were 5.6 % peroxyacetic acid and 26.5 % H_2O_2. The treatment time periods for this chemical were assigned at 5 minutes, 15 minutes, 30 minutes, and 1 hour.

Table 2. Different sanitizers, their trade name, active ingredients, and used levels.

Trade Name	Active ingredients	Use level	Source
Bi-Quat	Dimethyl ethylbenzy (5.1%) ammonium chloride Alkyl dimethyl benzyl (5.1%) ammonium chloride Ethanol (1.1%)	200 ppm	Birko Corp
10-Chlor	Sodium hypochlorite (<20%) Sodium hydroxide (<5%)	200 ppm & 1000 ppm	Birko Corp
Sterilex solution	1.Ultra Disinfectant Cleaner Hydrogen peroxide (5.5-7.2%) Alykl dimethyl ethyl benzyl ammonium chloride (2.5-3.5%) Alkyl (C12,C14,C16) Dimethyl Benzyl Ammonium Chloride (2.5-3.5%) 2. Ultra Activator Solution Sodium carbonate (4-8%) Potassium carbonate (4-8%) Tetrasodium ethylenediaminetetraacetate (3-7%)	5% & 10%	Sterilex Corporation

Decon7 solution	1.Quaternary ammonium chloride Benzyl-C12-C16 Alkyl Di-methyl Chlorides (5.5-6.5%) 2. Hydrogen peroxide (<8%) 3. Diacetin (30-60%)	5% & 10%	Decon ™ Seven Systems
KC-610	Peroxyacetic acid (5-6%) Hydrogen peroxide (25-58%) Acetic acid (5-10%)	500 ppm	Packers Chemical

Enumeration of residual viable cells. After treatment with sanitizers and neutralization using DE buffer, the lethality of sanitizers was quantified by microplate biofilm detachment assay (enzymatic detachment and plating). The test wells (treated with sanitizers and neutralized) were washed with Tris (0.05 M. pH 7.4) in a plate washer. Then 200 µl of trypsin (426.4 U/ml) was added into the wells and incubated for an hour at 37º C. The enzyme added wells were then harvested and the liquid was plated on Tryptic Soy Agar (TSA) plates. The plates were then incubated for 24 hours at 30º C and enumerated for residual viable cells the next day.

Scanning Electron Microscopy (SEM)

SEM of *L. monocytogenes*. The adherent strain *Listeria monocytogenes* 99-38 (10^4 log cfu/ml; 300 µL) was allocated into wells of Millicell EZ Slide 8 well glass slide (Millipore Sigma, Sheboygan Falls, WI) (Fig. 4) sealed with parafilm to avoid evaporation and incubated at 30º C. The media (BHI) in the wells was changed each day after washing with Tris (0.05 M; pH 7.4) and the process was continued for 7 days to get a 7 day old mature biofilm in the wells. The wells were then assigned different treatments based on the sanitizer lethality assays.

Figure 4. Millicell EZ Slide 8 well glass slide.

After treatment, the slides were washed one final time with Tris buffer. A standard protocol (listed below) provided by Oklahoma State University's Electron Microscopy lab was used to fix, dry and coat the samples before imaging.

1. The cells were fixed for 2 hr. in 2.0% glutaraldehyde in 0.1M cacodylate buffer (21.4g sodium cacodylate brought to 500ml with dH_2O).

2. The sections were rinsed 3X in buffered wash (60ml of 0.2M cacodylate buffer, 140ml of dH_2O, and 12.3g of sucrose; 15'/rinse).

3. The adherent cells were again fixed for 1 hr. in 1% aqueous OsO_4 (room temperature).

4. They were then rinsed 3X in buffered wash (15'/rinse).

5. It was followed by dehydration in ethanol of different concentrations: 50%, 70%, 90%, 95%, and 100% (3X). (15'/step)

6. Then the slide was subjected to critical point dry (CPD) or washed 2X for 5 min with HMDS (Hexamethyldisilazane).

7. Silver paint or double-sticky tape was used to mount on stubs.

8. Then the gold-palladium (Au-Pd) coating of the attached cells was performed.

9. The sections on slide were then visualized or stored in a dust-free, dry area, such as a desiccator to view later. The qualitative visualization of the effects of different treatments on *L. monocytogenes* 99-38 biofilms was done by using FEI Quanta 600 FEG scanning electron microscope at Electron Microscopy Core Facility.

Experimental Design and Statistical Analysis.

Each trial was performed in triplicate replication. All data were presented as the mean of triplicate replications and standard deviation of the mean were represented by error bars. Statistical analysis was done by using one way analysis of variance (ANOVA) and Holm-Sidak test for pairwise multiple comparisons to determine significant differences ($P < 0.05$) among multiple treatment means and standard deviations. For the sanitizers' treatment over time periods, one way ANOVA with repeated measures was carried out to see significant differences on effect of sanitizers against the biofilms.

CHAPTER IV

RESULTS

Microplate biofilm adherence assay optimization

Fluorescent substrate selection. Using *L. monocytogenes* 99-38 as a test organism, we found that 5,6-carboxy fluorescein diacetate (5,6-CFDA) and 5,6-carboxy fluorescein diacetate, succinimidyl ester (5,6-CFDA, SE) had significantly different levels of relative fluorescence units (RFU) (Fig. 5). The 5,6-CFDA dye gave higher fluorescent signals with *L. monocytogenes* biofilms (32,724 RFU) than the 5,6-CFDA-SE dye (7,186 RFU). The difference in mean RFU obtained by the dyes were statistically significant ($p<0.05$).

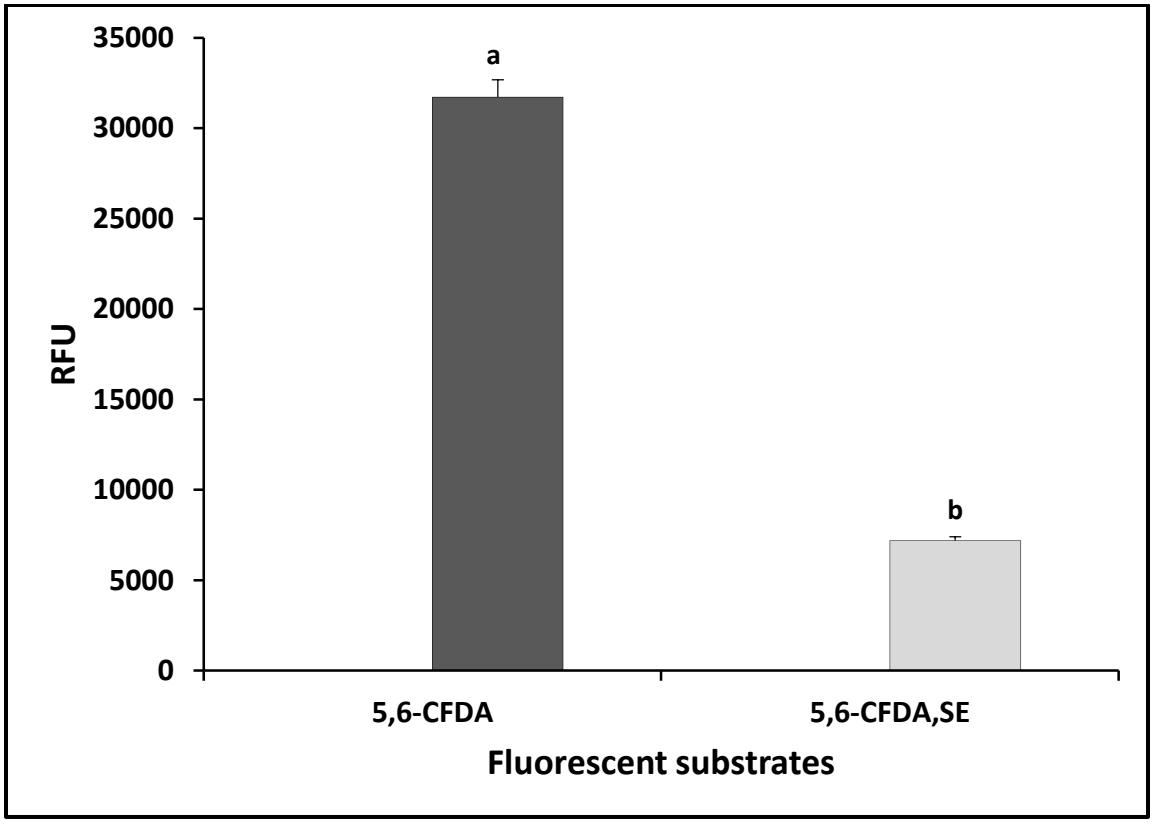


Figure 5. Examination of fluorescence signals obtained from adherence of *L. monocytogenes* 99-38 using fluorescence substrates 5,6-CFDA and 5,6-CFDA, SE. The fluorescence signal is reported as relative fluorescence units (RFU). Data bars represent the means of triplicate replications and error bars represent standard deviation of the means. Bars with different letters represent significant differences in the means ($p < 0.05$)

Microplate washing time optimization. The preliminary experiment performed to optimize the wash times for our microplate assay showed that 3 times washing of 48 hours old *L. monocytogenes* 99-38 biofilms with Tris buffer prior to enzymatic detachment yielded 8.11 log CFU/ml of viable cells (Fig. 6). The levels of cellular detachment were 8.0 log CFU/ml and 7.94 CFU/ml, respectively, with 9 and 15 buffer wash time periods. The detachment levels with enzyme after 3 times wash and 15 times wash were significantly different ($p<0.05$) but levels of detachment of cells between 3 times and 9 times wash and between 9 times and 15 times wash were not significantly different.

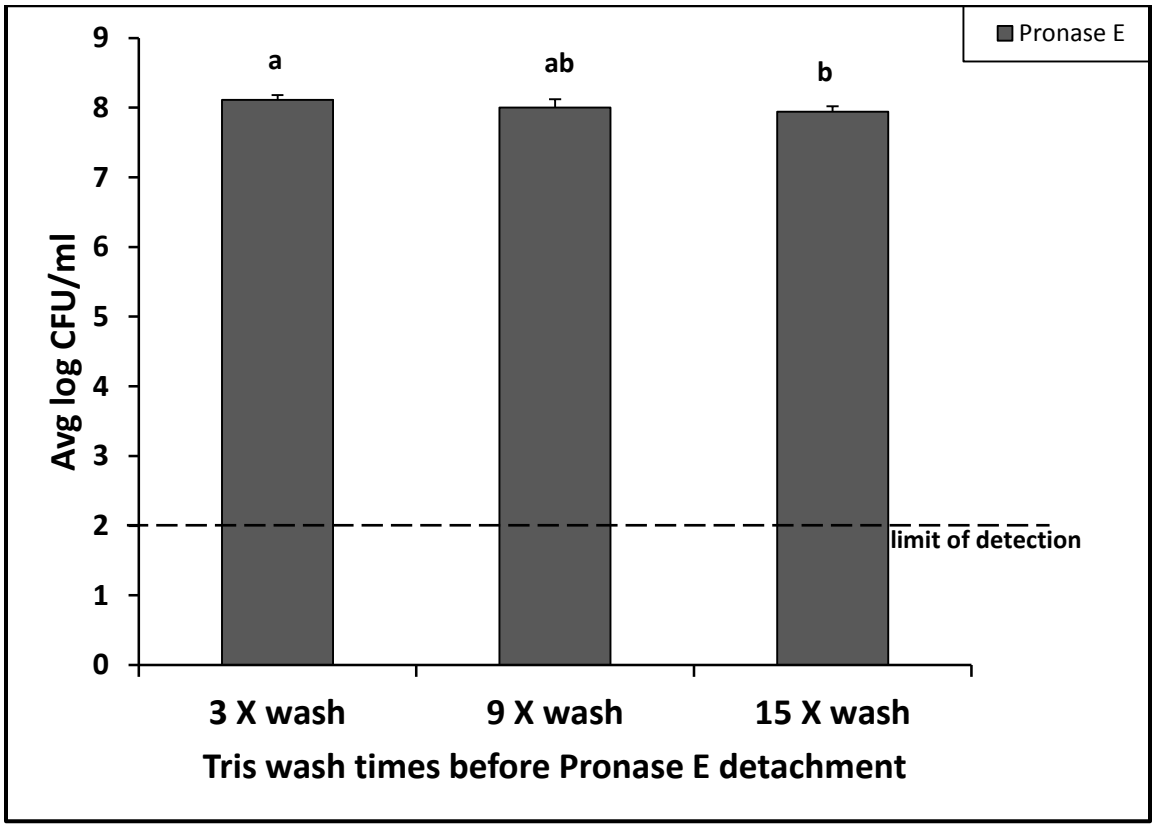


Figure 6. Optimization of wash times for enzymatic detachment and enumeration of 2 days old L. monocytogenes 99-38 biofilms. The detachment levels are represented by average log CFU/ml. Data bars represent the means of triplicate replications and error bars represent standard deviation of the means. Bars with different letters represent significant differences in the means ($p < 0.05$); bars with the same lowercase letters are not significantly different ($p > 0.05$).

Adherence Characteristics of Pathogenic Microorganisms Confirmation (*L. monocytogenes*) and screening (*E. coli* O157:H7, *Salmonella* serovar) of strongly adherent strains using the microplate fluorescence assay.

***Listeria monocytogenes*.** *L. monocytogenes* strain 99-38 was confirmed as strongly adherent and CW 35 as weakly adherent strains using the microplate fluorescence adherence assay. The statistical analysis of mean RFU levels obtained from the microplate adherence assay of the strains showed significant difference ($p<0.05$) between themselves and also with the control (Fig. 7).

***Escherichia coli* 0157:H7.** *Escherichia coli* 0157:H7 strains 2-RR_2, 2-ML_2 and ATCC 43888 demonstrated low fluorescence levels in the fluorescence adherence assay and were screened as weakly adherent while *E. coli* O157:H7 F-4546 gave significantly higher levels and was considered as strongly adherent strain. The group of strains categorized as weakly adherent and strongly adherent had significantly different ($p<0.05$) means. Similarly, the mean RFU levels obtained from strongly adherent *E. coli* O157:H7 F-4546 was significantly different from control ($p<0.05$) but those from weakly adherent ones were not (Fig. 8).

***Salmonella* spp.** All of the *Salmonella enterica* serovars evaluated for their adherence capability showed lower RFU values compared to strongly adherent strains of *L. monocytogenes* and *E. coli* O157:H7 (Fig. 10). However, *Salmonella* Montevideo FSIS051 which gave the highest mean RFU reading (5010.7) and was selected for further analysis as it had a significantly different ($p<0.05$) mean RFU value than the other serovars (Fig. 9).

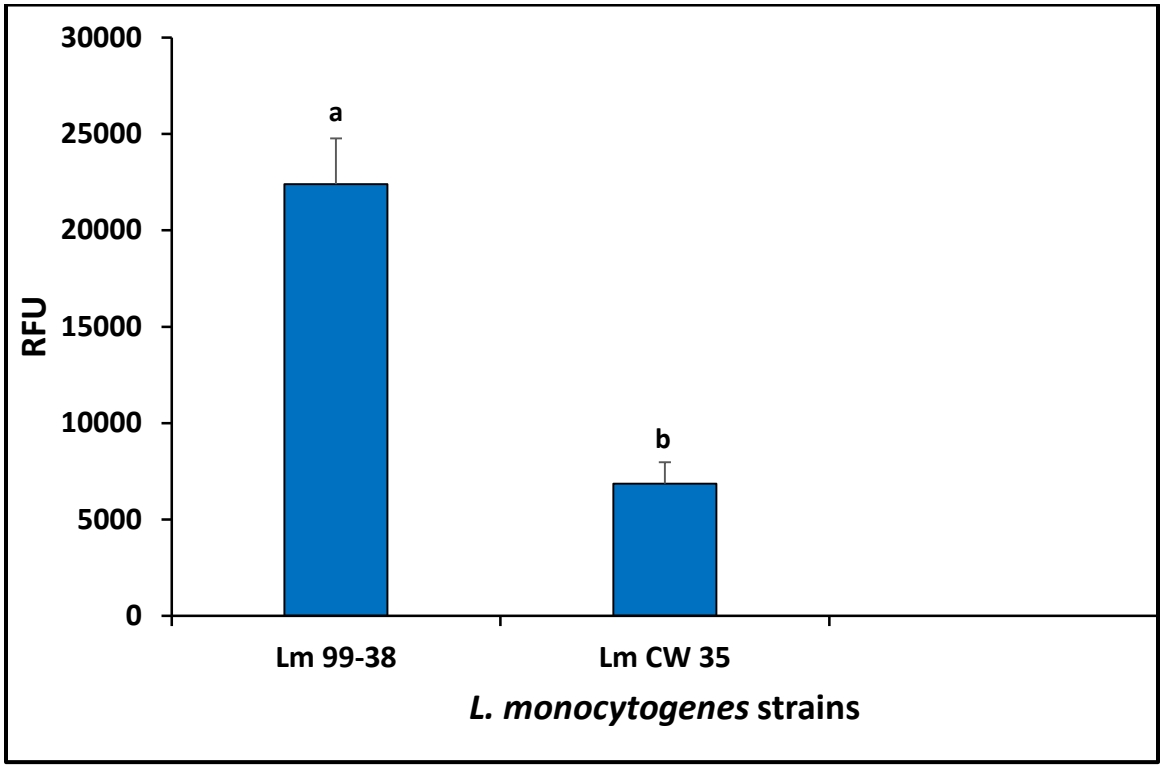


Figure 7. Microplate fluorescence adherence assay of L. monocytogenes 99-38 and CW35 vs Tris buffer (blank) using 5,6-CFDA and 3 wash cycles. The fluorescence signal is reported as relative fluorescence units (RFU). Data bars represent the means of triplicate replications and error bars represent standard deviation of the means. Bars with different letters represent significant differences in the means ($p < 0.05$).

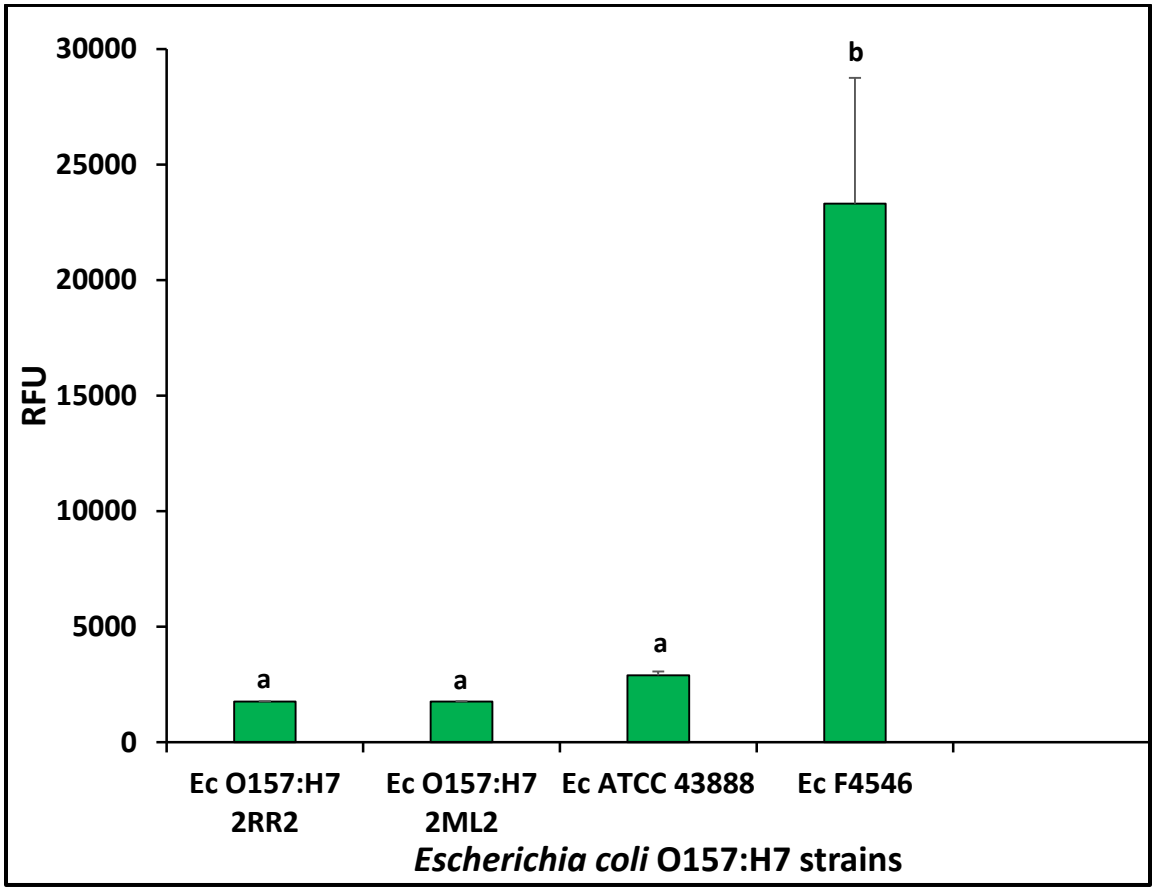


Figure 8. Microplate fluorescence adherence assay of *E. coli* O157:H7 (2RR2, 2ML2, ATCC 43888, F4546) using 5,6-CFDA and 3 wash cycles. The fluorescence signal is reported as relative fluorescence units (RFU). Data bars represent the means of triplicate replications and error bars represent standard deviation of the means. Bars with different letters represent significant differences in the means ($p < 0.05$); bars with the same lowercase letters are not significantly different ($p > 0.05$).

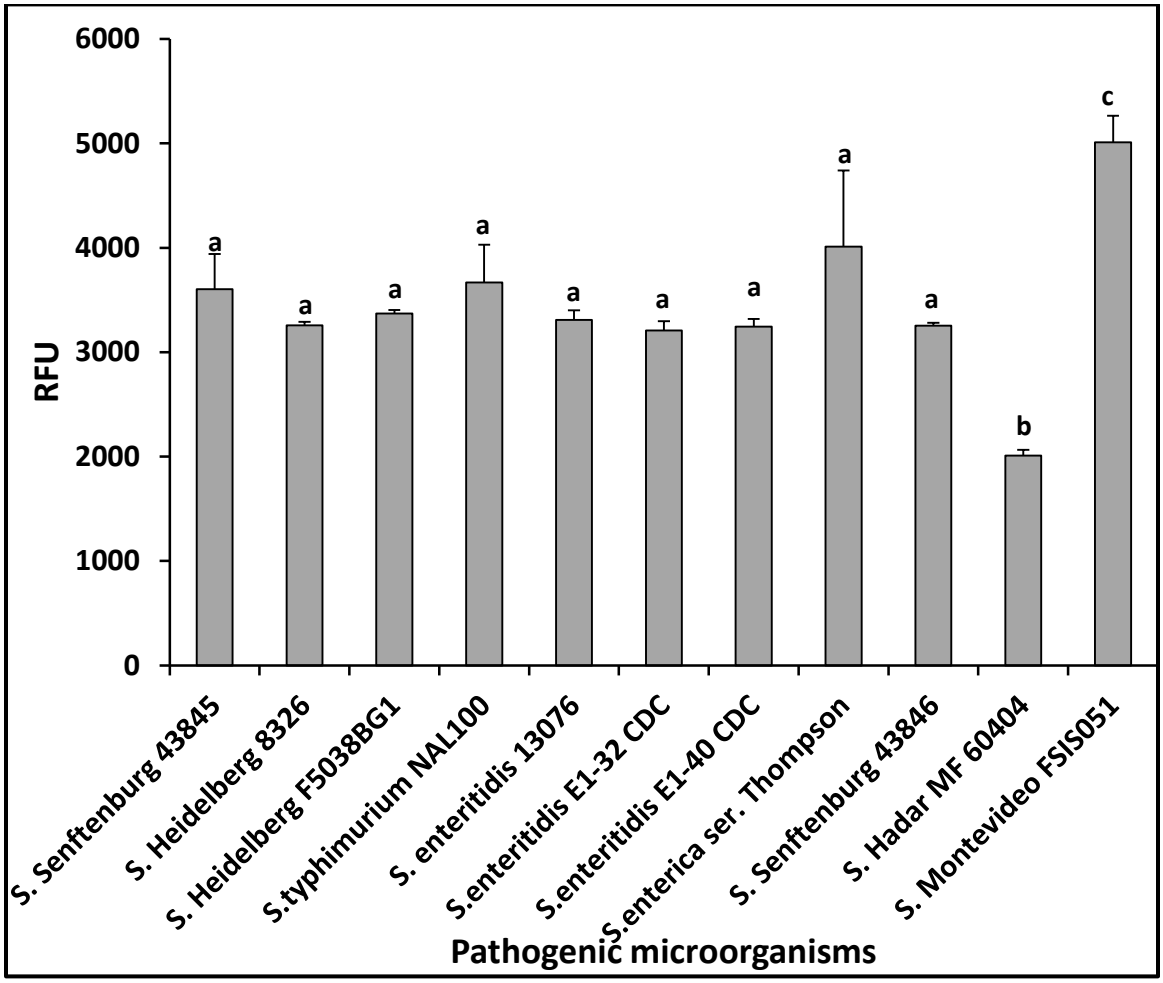


Figure 9. Microplate fluorescence adherence assay of *Salmonella* serovars using 5,6-CFDA and 3 wash cycles. The fluorescence signal is reported as relative fluorescence units (RFU). Data bars represent the means of triplicate replications and error bars represent standard deviation of the means. Bars with different letters represent significant differences in the means ($p < 0.05$); bars with the same lowercase letters are not significantly different ($p > 0.05$).

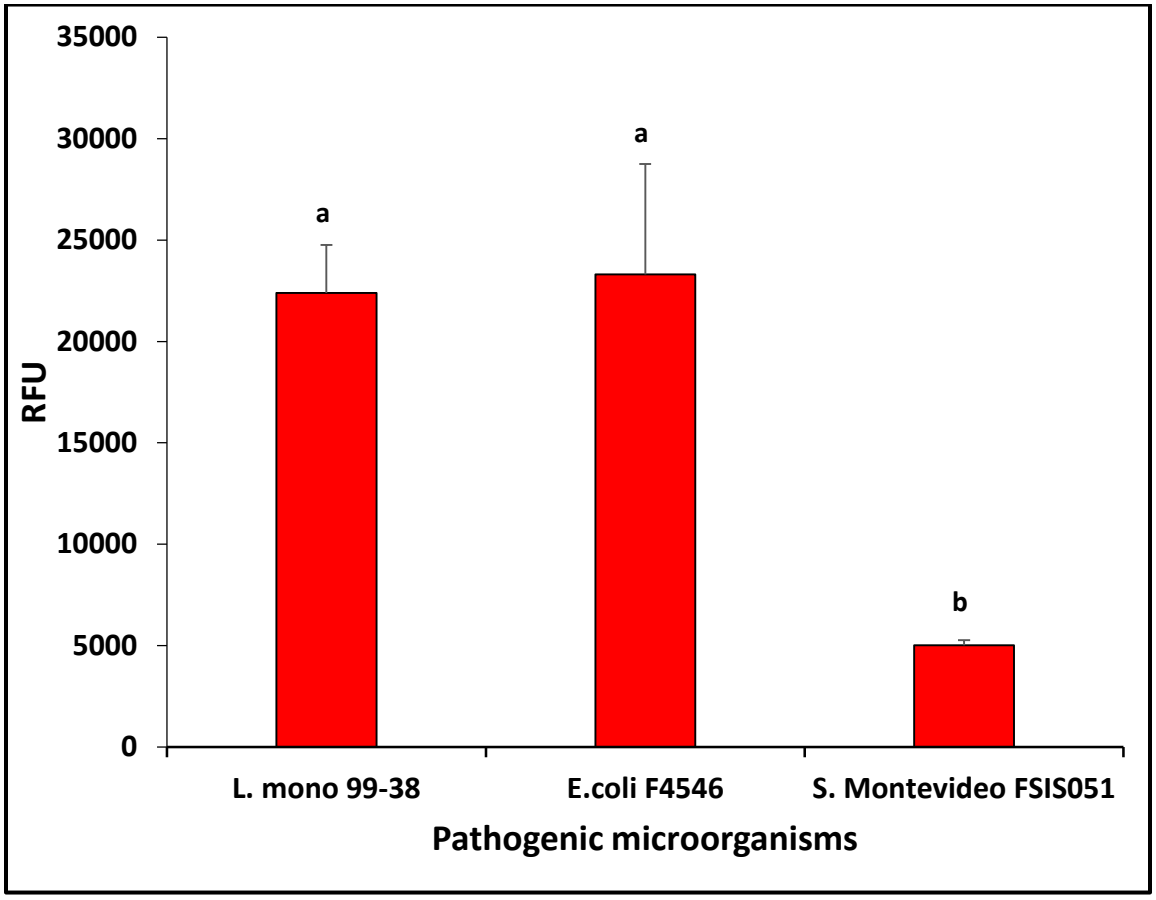


Figure 10. Microplate fluorescence adherence assay comparison of *L. monocytogenes* 99-38, *E. coli* F4546, and *S.* Montevideo FSIS051. The fluorescence signal is reported as relative fluorescence units (RFU). Data bars represent the means of triplicate replications and error bars represent standard deviation of the means. Bars with different letters represent significant differences in the means ($p < 0.05$); bars with the same lowercase letters are not significantly different ($p > 0.05$).

Enzymatic Detachment of Biofilms

Detachment levels over time. The experiment to check the levels of detachment of cells from biofilms over time resulted in a trend which showed slight increases in the number of viable recoverable cells with increased age of biofilms. A day-old biofilm of *L. monocytogenes* 99-38 when detached using a protease enzyme gave average plate counts of 7.24 log CFU/ml. The means of enumerated levels of cells on the 2^{nd}, 3^{rd}, 4^{th} and 5^{th} days were in increasing order of 7.68 log CFU/ml, 7.91 log CFU/ml, 7.92 log CFU/ml and 8.08 log CFU/ml respectively (Fig. 11) and all were significantly different ($p< 0.05$) in comparison to the detachment levels from the prior day biofilm (except the 4^{th} day biofilm).

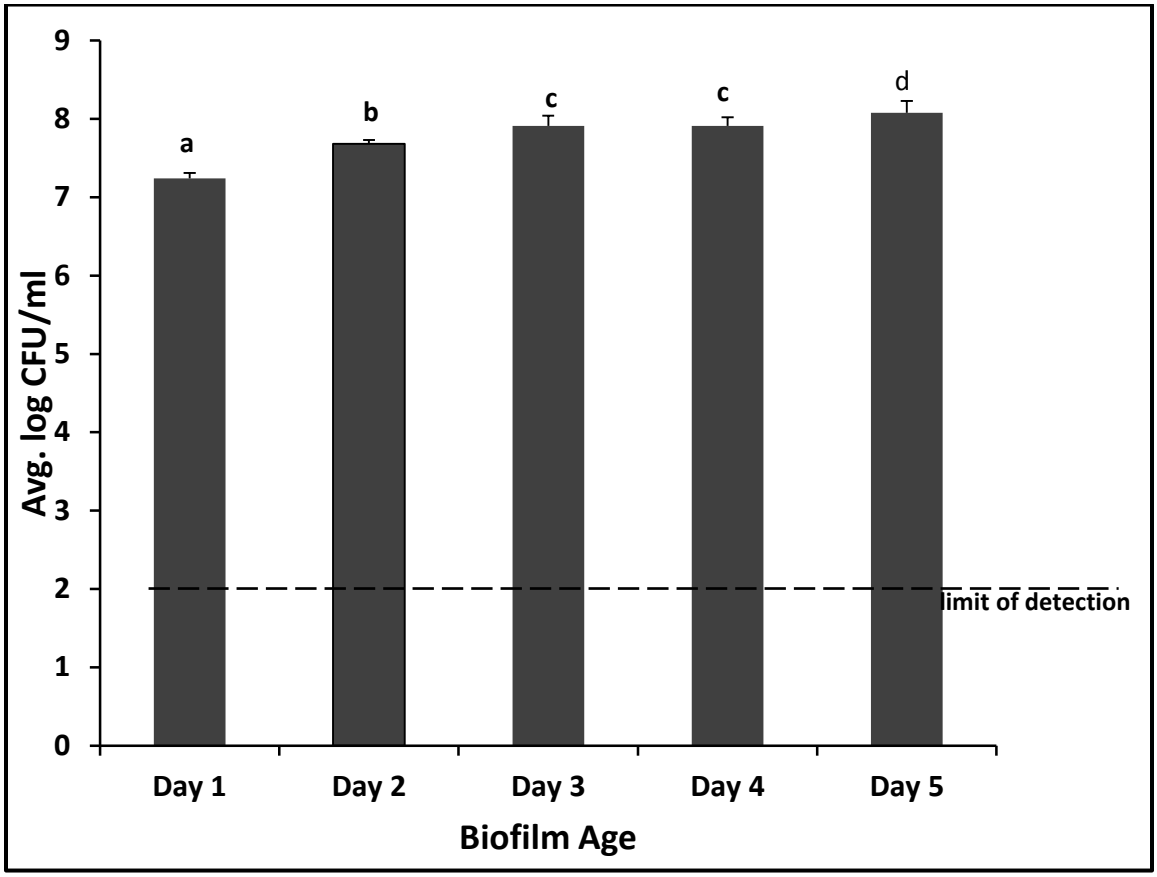


Figure 11. Enumeration of *L. monocytogenes* 99-38 biofilm levels over time. Planktonic cells were washed and replaced daily with fresh sterile media for up to 5 days. Enumeration was performed after detachment with pronase E. The detachment levels are represented as average log CFU/ml. Data bars represent the means of triplicate replications and error bars represent standard deviation of the means. Bars with different letters represent significant differences in the means ($p < 0.05$); bars with the same lowercase letters are not significantly different ($p > 0.05$).

Detachment of bacterial cells using enzymes. Various enzymes used for detachment and enumeration of microbial biofilms showed different efficacies for different pathogens. The multiple pairwise comparison among enzymatic treatment means yielded varying results as shown in graphs below (Fig. 12, 13, 14, 15). The proteases such as pronase E (500 U/ml), bax protease (12.5 µl per 1ml Tris) and trypsin (1485.9 U/ml and 426.4 U/ml) showed levels of detachment of *E. coli* 0157:H7 F4546 and *L. monocytogenes* 99-38 at above 8 log CFU/ml (Fig. 12, 13, 15) whereas enzymes such as cellulase, lipase, and papain (all at 500 U/ml) also showed more than 7 log CFU/ml of detachment (Fig. 12, 13, 15). The number of detached cells of *S.* Montevideo FSIS051 by selected enzymes was between 7 log CFU/ml and 8 log CFU/ml (Fig. 14). Trypsin (426.4 U/ml) was selected for further use in subsequent assays due to its high detaching ability (Fig. 15), low cost and ease in preparing working solution.

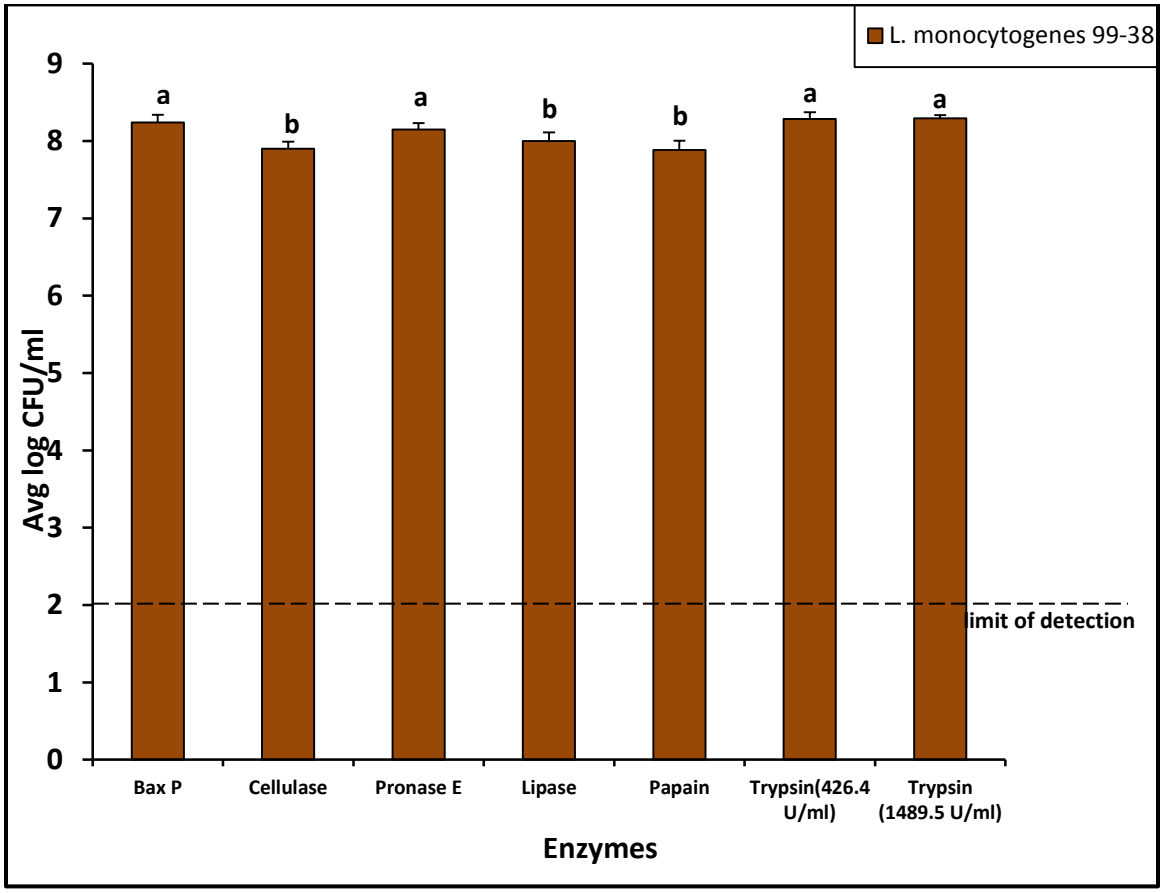


Figure 12. Enumeration of *L. monocytogenes* 99-38 biofilm levels detached after treatment with various enzymes; Bax P, Cellulase, Pronase E, Papain, Trypsin, and Lipase. Planktonic cells were washed and replaced daily with fresh sterile media for up to 7 days. The levels of detachment are represented in terms of average log CFU/ml. Data bars represent the means of triplicate replications and error bars represent standard deviation of the means. Bars with different lowercase letters represent significant differences in the means ($p < 0.05$); bars with the same lowercase letters are not significantly different ($p > 0.05$).

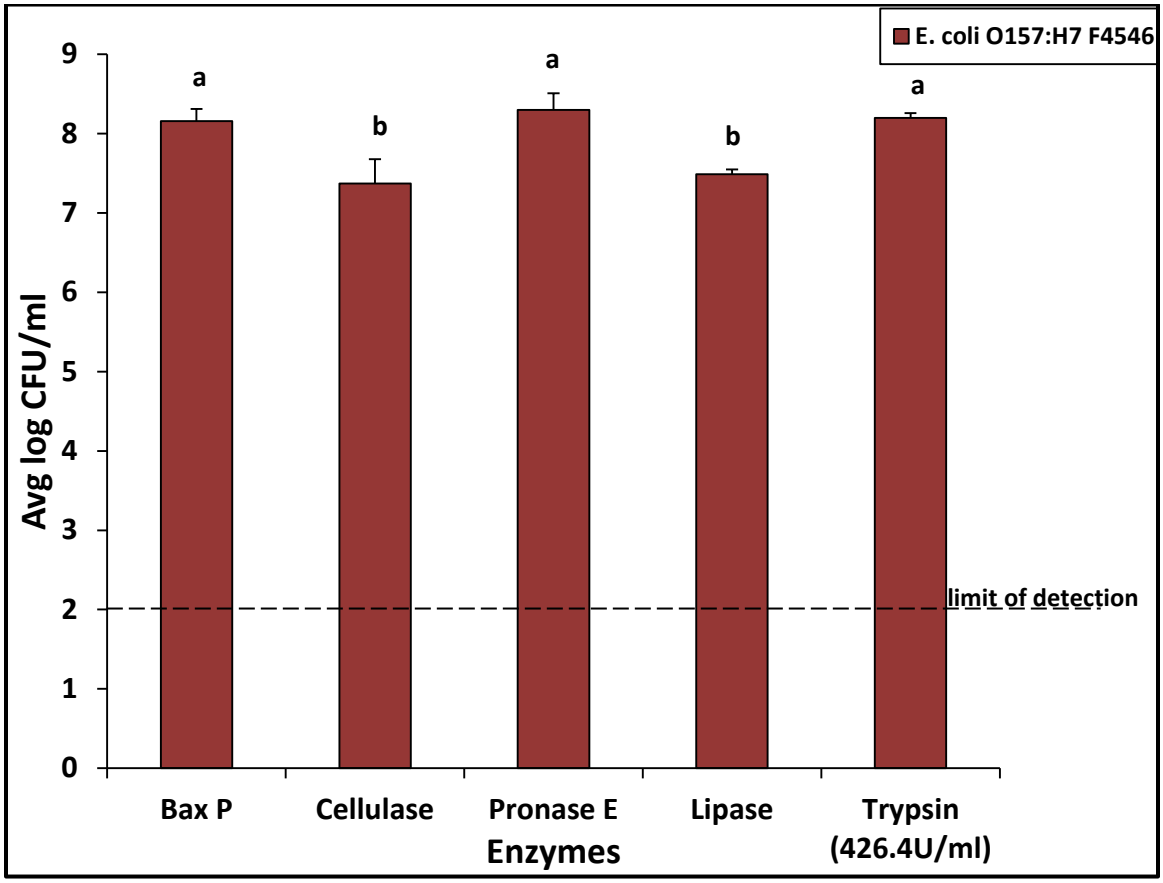


Figure 13. Enumeration of *E. coli* O157:H7 F4546 biofilm levels detached after treatment with various enzymes; Bax P, Cellulase, Pronase E, Lipase, and Trypsin. Planktonic cells were washed and replaced daily with fresh sterile media for up to 7 days. The levels of detachment are represented in terms of average log CFU/ml. Data bars represent the means of triplicate replications and error bars represent standard deviation of the means. Bars with different lowercase letters represent significant differences in the means ($p < 0.05$); bars with the same lowercase letters are not significantly different ($p > 0.05$).

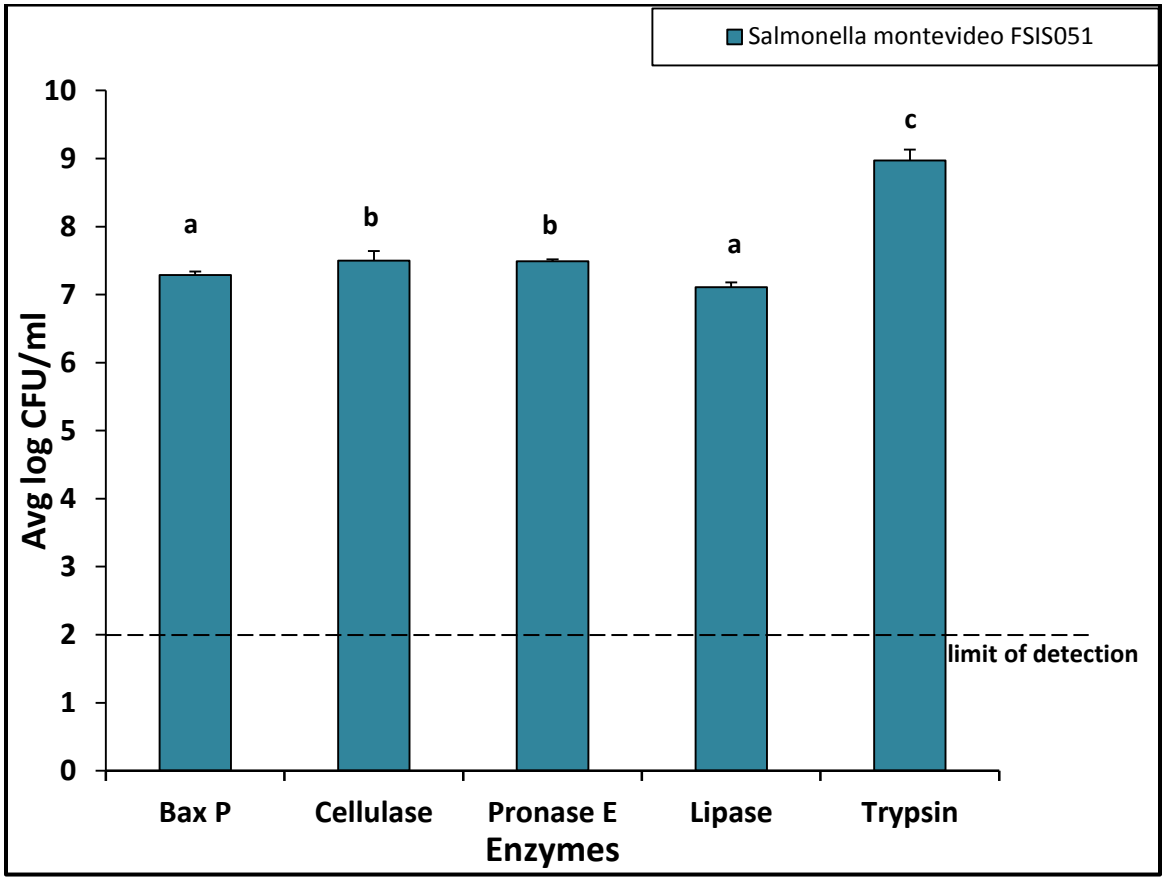


Figure 14. Enumeration of *Salmonella* Montevideo FSIS051 biofilm levels detached after treatment with various enzymes; Bax P, Cellulase, Pronase E, Lipase, and Trypsin. Planktonic cells were washed and replaced daily with fresh sterile media for up to 7 days. Data bars represent the means of triplicate replications and error bars represent standard deviation of the means. Bars with different lowercase letters represent significant differences in the means ($p < 0.05$); bars with the same lowercase letters are not significantly different ($p > 0.05$).

Sanitizers treatment and lethality

Sanitizer: Bi-Quat. Bi-Quat (200 ppm) treatment of a 7-day old *L. monocytogenes* 99-38 biofilm (washed and media replaced daily) for 15 minutes showed a 5 log CFU/ml reduction (Fig. 15). When the treatment period was increased to 30 minutes, the viable cell count was below the limit of detection (LOD) of 2 log CFU/ml. *E. coli* 0157:H7 F4546 biofilms under action of Bi-Quat 200 ppm were less sensitive to Bi-Quat, showing only a log CFU/ml reduction after 2 hours of immersion in the sanitizer. The biofilms of *Salmonella* Montevideo FSIS051 were also insensitive to Bi-Quat, being reduced from 9.19 log CFU/ml to 8.19 log CFU/ml in 2 hours, (1 log CFU/ml reduction). The statistical significance between the different organisms (repeated measures one-way ANOVA) for treatment with Bi-Quat (200 ppm) sanitizers are shown in Figure 15.

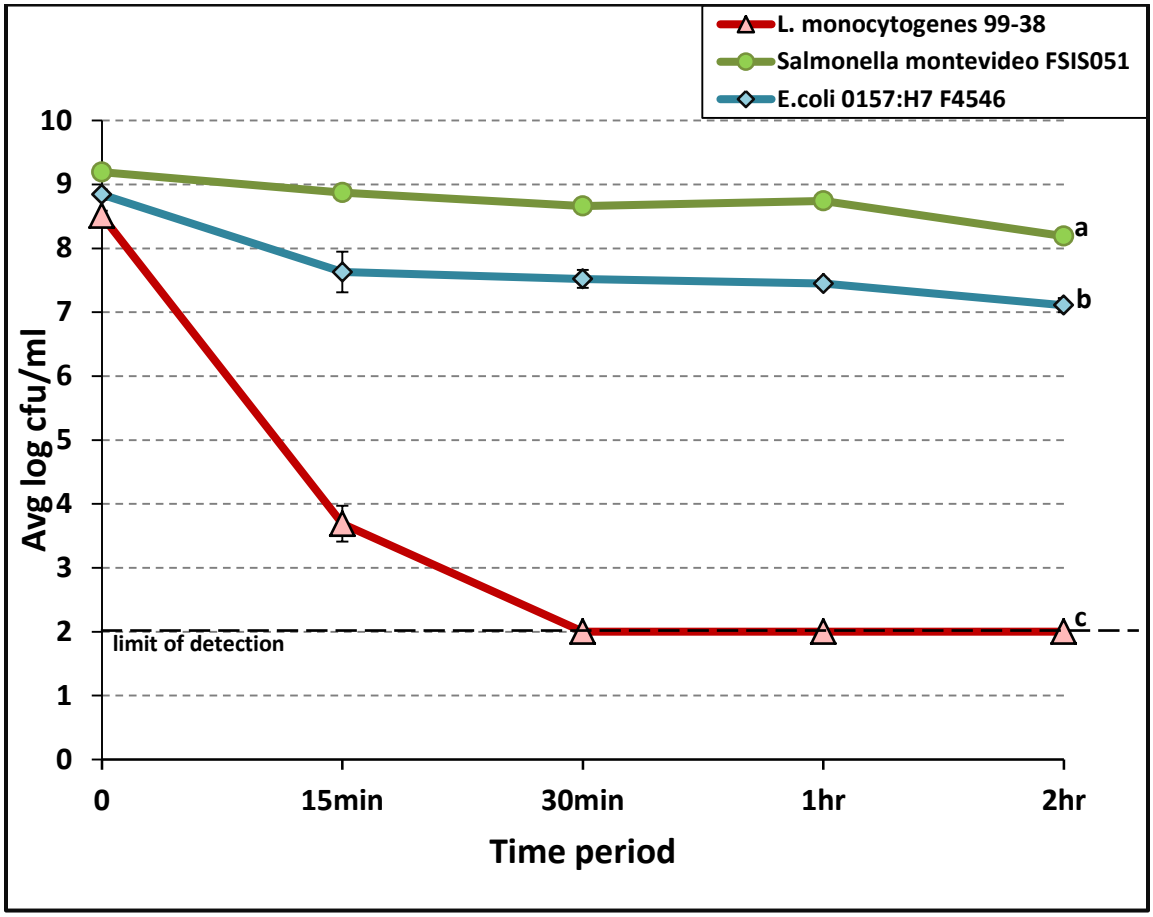


Figure 15. Lethality of Bi-Quat (200 ppm) on biofilms of *L. monocytogenes* 99-38, *E. coli* O157:H7 F4546, and *Salmonella* Montevideo FSIS051. The error bars represent the standard deviation from mean. The treatments with different letters represent significant differences in the trend ($p < 0.05$). The limit of detection is 2 log CFU/ml.

Sanitizer: 10-Chlor. Biofilms of *L. monocytogenes* 99-38, *E. coli* F4546, and *Salmonella* Montevideo FSIS051 were treated with 200 ppm of chlorine-based sanitizer (10-chlor) for upto 60 min using the microplate adherence assay format. Biofilms from all 3 organisms showed a similar rate of decline of approximately 2-logs over 60 min of treatment (Fig. 16). An increase in the concentration of 10-chlor by 5-fold (i.e. 1000 ppm) helped to achieve > 6-log reduction, reducing biofilm levels below the limit of detection of 2 log CFU/ml for *L. monocytogenes* (15 min) and *E. coli* (60 min), however *Salmonella* was still largely unaffected (~ 1.5 log reduction in 60 min) by this chlorine-based sanitizer (Fig. 17). The statistical significance between the different organisms (repeated measures one-way ANOVA) for each treatment with 10-chlor sanitizers are shown in Figures 16 and 17.

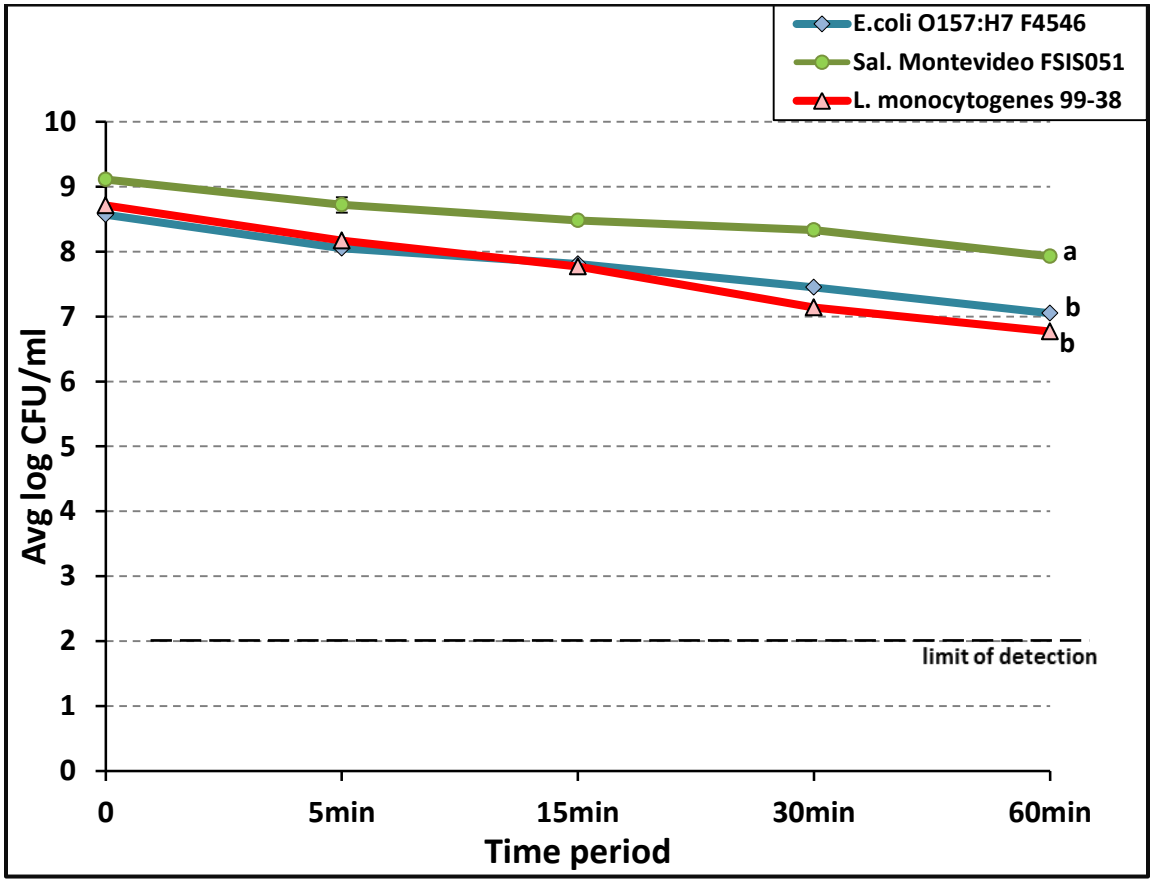


Figure 16. Lethality of 10-Chlor (200 ppm) on biofilms of *L. monocytogenes* 99-38, *E.coli* O157:H7 F4546 and *Salmonella* Montevideo FSIS051. The error bars represent the standard deviation from mean. The treatments with different letters represent significant differences in the trend ($p < 0.05$); treatments with the same lowercase letters are not significantly different ($p > 0.05$). The limit of detection (2 log CFU/ml) is represented by the dashed line.

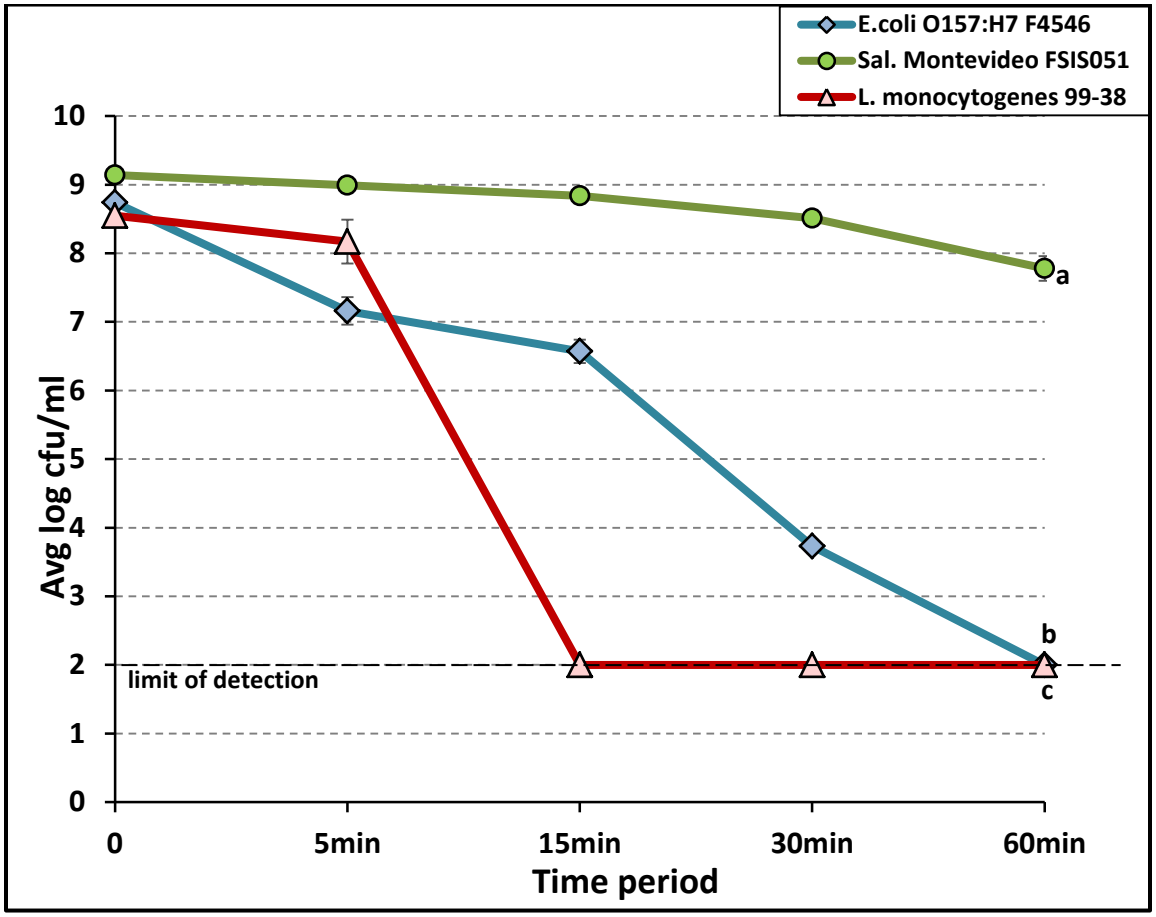


Figure 17. Lethality of 10-Chlor (1000 ppm) on biofilms of *L. monocytogenes* 99-38, *E.coli* O157:H7 F4546 and *Salmonella* Montevideo FSIS051. The error bars represent the standard deviation from mean ($p < 0.05$). The treatments with different letters represent significant differences in the trend ($p < 0.05$); treatments with the same lowercase letters are not significantly different ($p > 0.05$). The limit of detection (2 log CFU/ml) is represented by the dashed line.

Sanitizer: Sterilex. Sterilex sanitizers (5%) were successful in eliminating *L. monocytogenes* 99-38 biofilms (<2 log CFU/ml) with just 2.5 minutes of treatment period. The time period for inactivation of biofilms was reduced to just 1 minute when the sterilex solutions were used at the concentration of 10%. In the same way, 20 minutes of treatment of *E. coli* O157:H7 F4546 biofilms with Sterilex solution (5%) reduced the viable cell counts by around 3 log CFU/ml (Fig. 18). However, when the concentration was increased to 10%, same 20 minutes of treatment with Sterilex solutions was sufficient to inactivate biofilms and more than 6 log CFU/ml reduction was achieved. In the case of *S.* Montevideo FSIS051 biofilms, sterilex solution (5%) reduced the initial cell population of 9.16 log CFU/ml to 8.97 log CFU/ml, 7.65 log CFU/ml, 7.55 log CFU/ml and 7.46 log CFU/ml in 2.5 minutes, 5 minutes, 10 minutes and 20 minutes of treatment time respectively (Fig. 18). The 10% sterilex treatment showed 2 log CFU/ml reductions of *Salmonella* in 20 minutes (Fig. 19). The statistical significance between the different organisms (repeated measures one-way ANOVA) for each treatment with Sterilex sanitizers are shown in Figures 18 and 19.

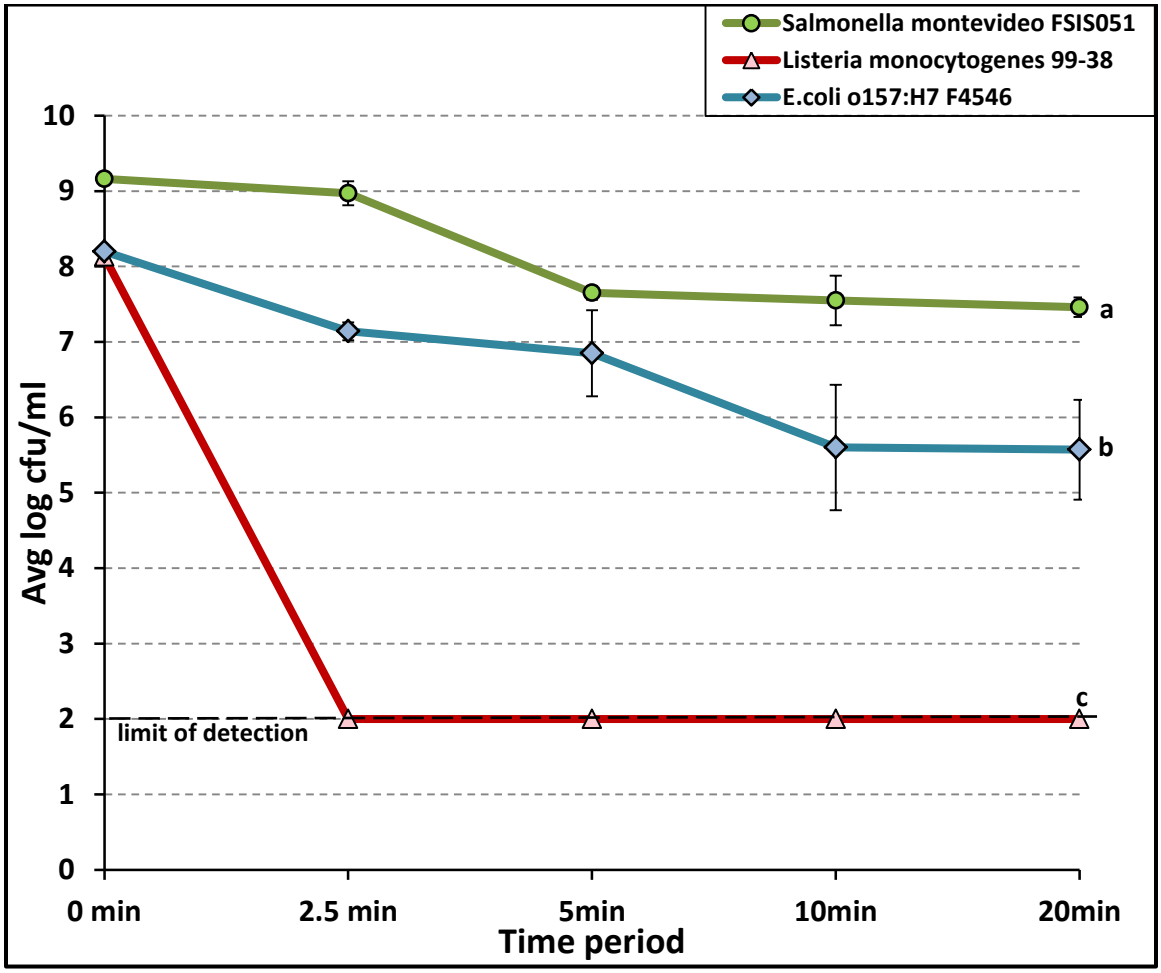


Figure 18. Lethality of Sterilex (5%) on biofilms of *L. monocytogenes* 99-38, *E.coli* O157:H7 F4546 and *Salmonella* Montevideo FSIS051. The error bars represent the standard deviation from mean. The treatments with different letters represent significant differences in their trend ($p < 0.05$). The minimum level of detection (2 log CFU/ml) is represented by the dashed line.

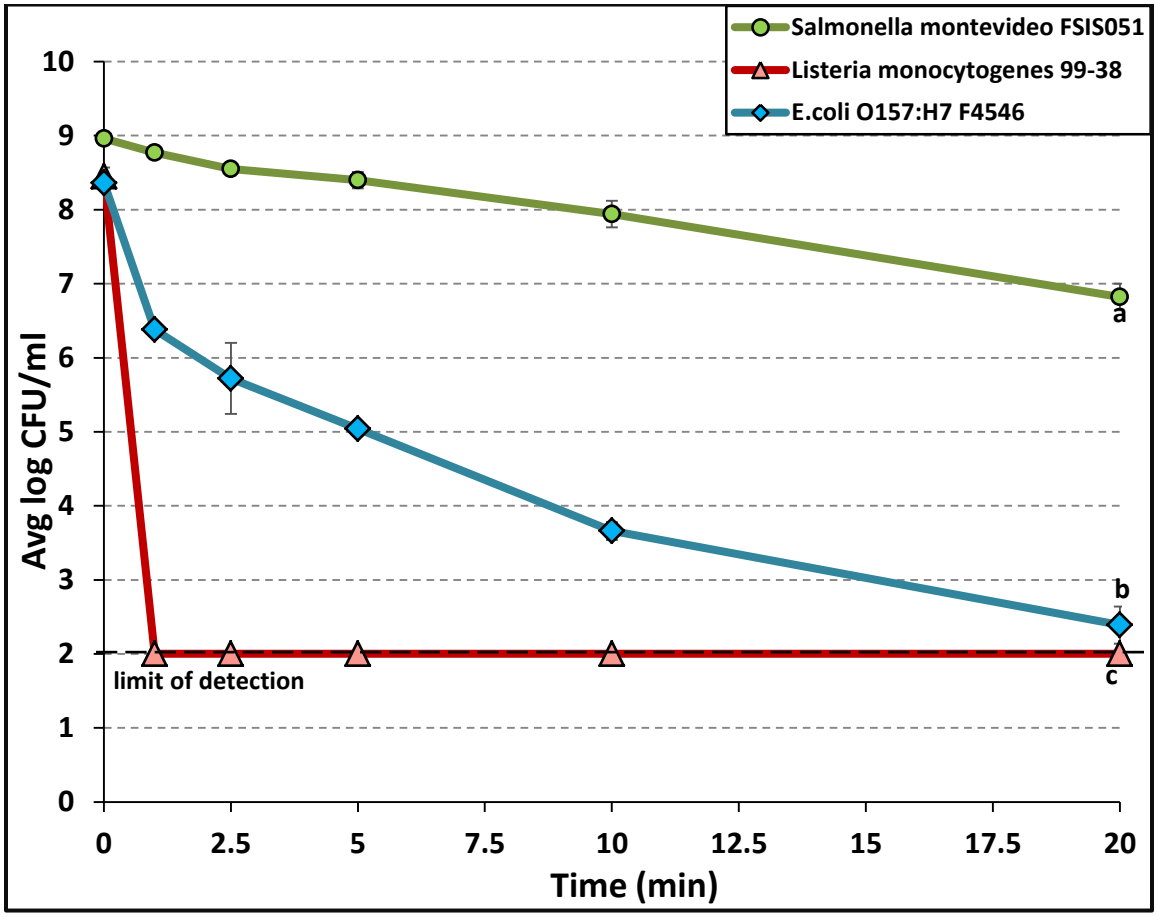


Figure 19. Lethality of Sterilex (10%) on biofilms of *L. monocytogenes* 99-38, *E.coli* O157:H7 F4546 and *Salmonella* Montevideo FSIS051. The error bars represent the standard deviation from mean. The treatments with different letters represent significant differences in their trend ($p < 0.05$). The limit of detection (2 log CFU/ml) is represented by the dashed line.

Sanitizer: Decon7. Decon7 sanitizers used at 5% and 10 % working strength were effective against *L.* monocytogenes 99-38 biofilm in microplate sanitizer lethality assay. At 5% working strength, Decon7 achieved a 6-log reduction within 2.5 min for both *L. monocytogenes* 99-38 and *E. coli* 0157:H7 whereas *Salmonella* Montevideo only observed a 3-log reduction after 20 min (Fig. 20). When Decon7 was used at 10% working strength, the sensitivity observed previously was even more pronounced and the limit of detection (>6-log reduction) was achieved within 1 min (Fig. 21). However, the lack of sensitivity of *Salmonella* Montevideo to 5% Decon7 (Fig. 20) was not observed with 10% Decon7 whereby the limit of detection (7-log reduction) was achieved within 2.5 min (Fig. 21). The statistical significance between the different organisms (repeated measures one-way ANOVA) for each treatment with Decon7 sanitizers are shown in Figures 20 and 21.

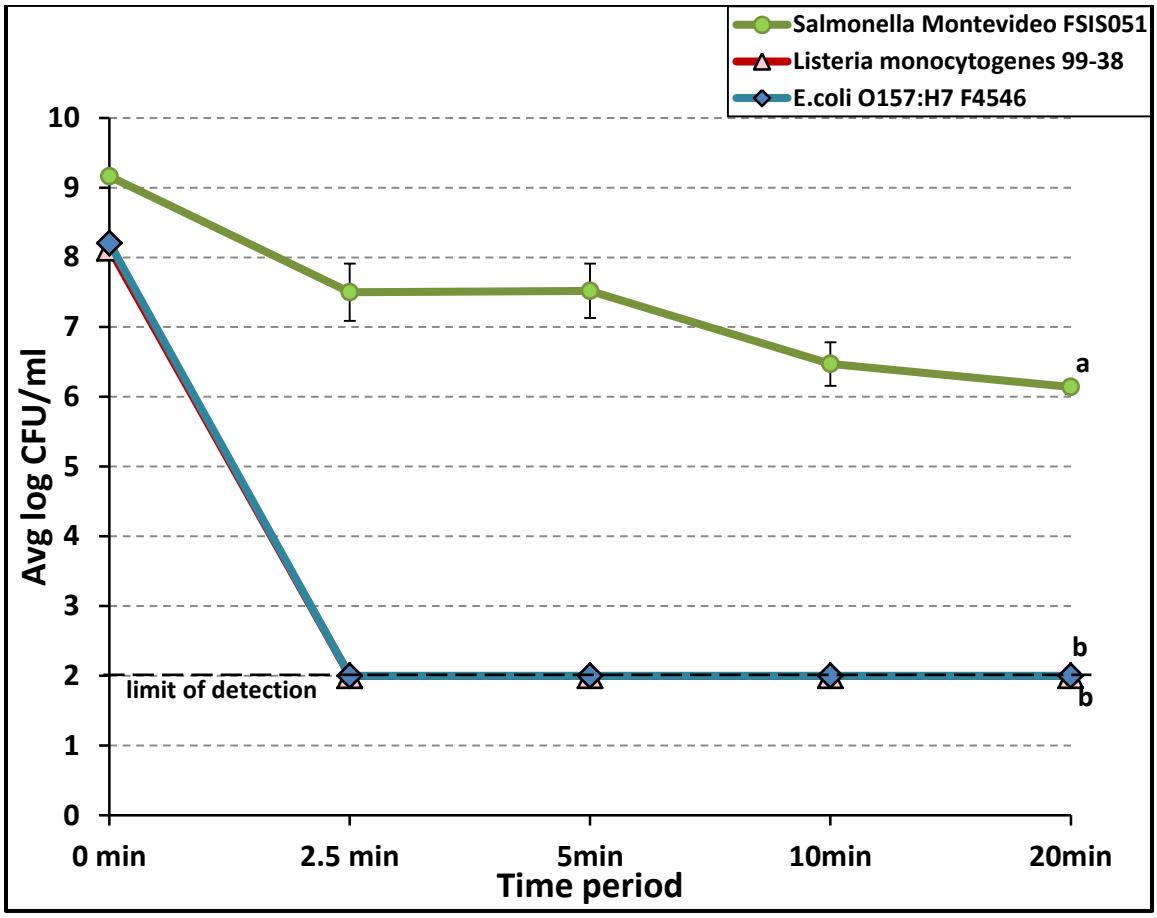


Figure 20. Lethality of Decon 7 (5%) on biofilms of *L. monocytogenes* 99-38, *E.coli* O157:H7 F4546 and *Salmonella* Montevideo FSIS051. The error bars represent the standard deviation from mean. The treatments with different letters represent significant differences in their trend ($p < 0.05$); treatments with the same lowercase letters are not significantly different ($p > 0.05$). The minimum level of detection (2 log CFU/ml) is represented by the dashed line.

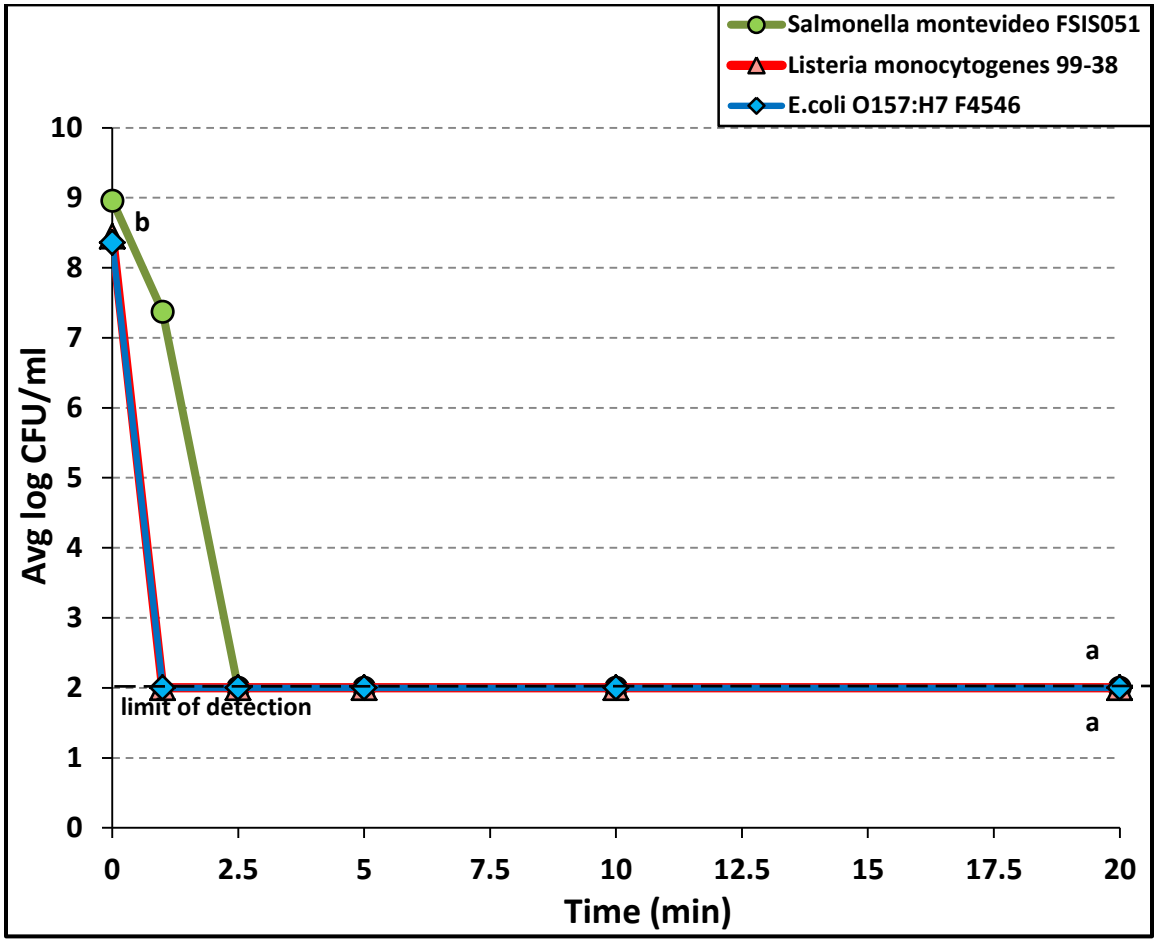


Figure 21. Lethality of Decon7 (10%) on biofilms of *L. monocytogenes* 99-38, *E.coli* O157:H7 F4546 and *Salmonella* Montevideo FSIS051. The error bars represent the standard deviation from mean. The treatments with different letters represent significant differences in their trend ($p < 0.05$); treatments with the same lowercase letters are not significantly different ($p > 0.05$). The limit of detection (2 log CFU/ml) is represented by the dashed line.

KC-610. The commercial sanitizer KC-610 is based on peroxyacetic acid (PAA). When used according to manufacturer's recommend actions (6.1 oz. in 6 gal; 500ppm active PAA) we observed a 6.65 log reduction of *L. monocytogenes* 99-38 and 6.37 log reduction of *E. coli* 0157:H7 F4546 within 5 min (Fig. 22). Again, as with the other sanitizers, *Salmonella* Montevideo was more resistant and required 30 min to reach the limit of detection (> 7-log reduction) (Fig. 22).

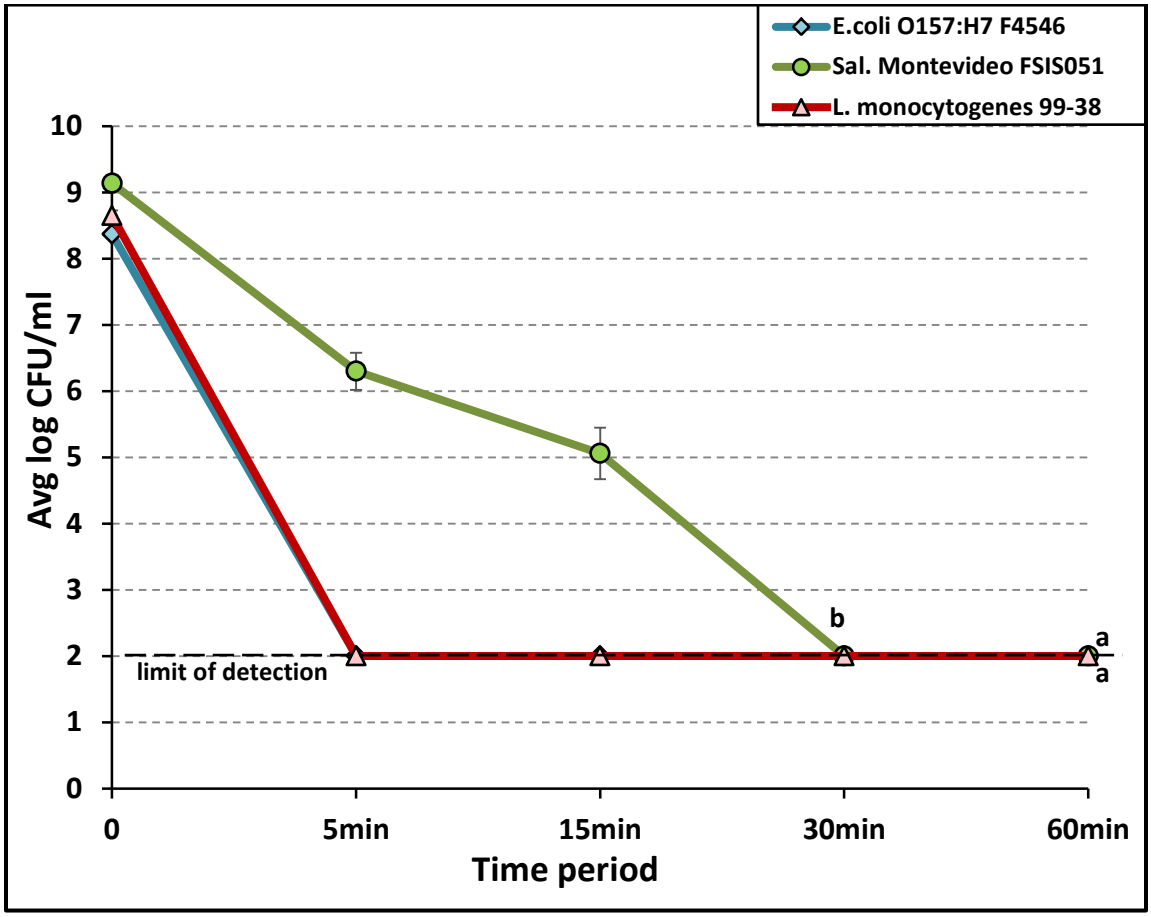


Figure 22. Lethality of KC-610 (6.1 oz. in 6 gal; 500 ppm active PAA) on biofilms of *L. monocytogenes* 99-38, *E.coli* O157:H7 F4546 and *Salmonella* Montevideo FSIS051. The error bars represent the standard deviation from mean. The treatments with different letters represent significant differences in their trend ($p < 0.05$); treatments with the same lowercase letters are not significantly different ($p > 0.05$). The limit of detection (2 log CFU/ml) is represented by the dashed line.

Scanning electron microscopy (SEM)

SEM of *Listeria monocytogenes*. Scanning electron microscopy enabled us to qualitatively visualize the lethality of *L. monocytogenes* 99-38 biofilms by sanitizers: Decon7, Sterilex and KC-610. As seen in Figure 23, *L. monocytogenes* 99-38 biofilms had comparatively reduced number of cells in sections treated with sanitizers than untreated control. The sections where enzyme (trypsin) was used in addition to sanitizers showed no or very few cells under SEM.

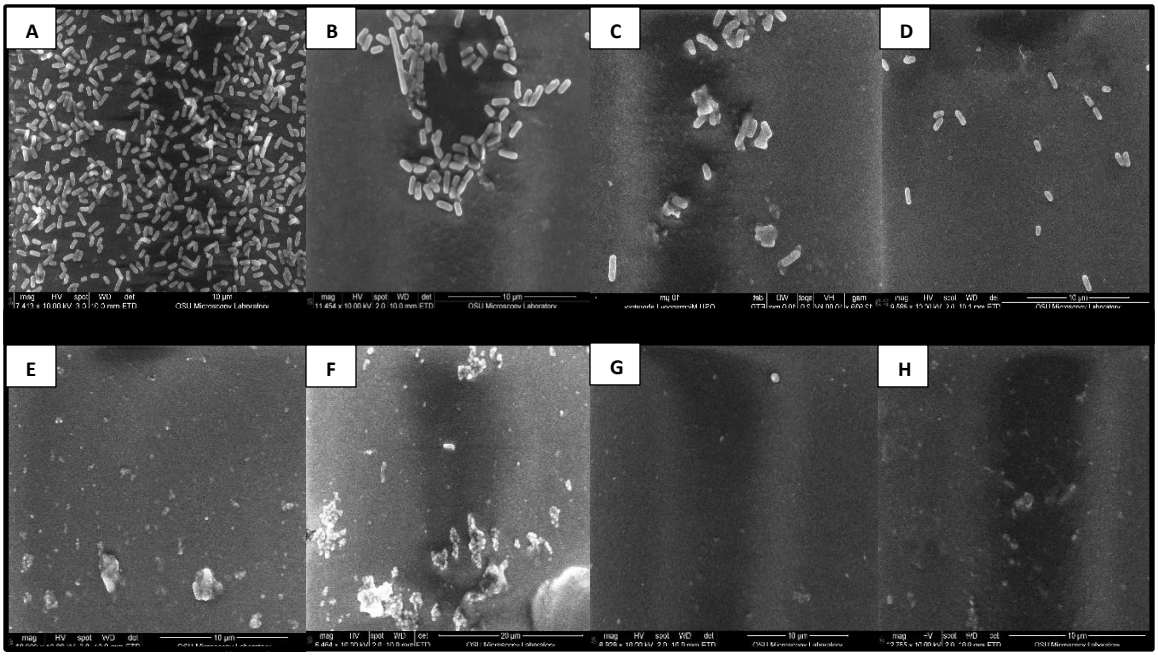


Figure 23. Observation of lethality of *L. monocytogenes* 99-38 by Decon7, Sterilex and KC-610 sanitizers. **Panel A:** *L. monocytogenes* control (untreated). **Panel B:** *L. monocytogenes* treated with Decon 7. **Panel C:** *L. monocytogenes* treated with Sterilex. **Panel D:** *L. monocytogenes* treated with KC-610. **Panel E:** *L. monocytogenes* + Trypsin. **Panel F:** *L. monocytogenes* + Decon7 + Trypsin. **Panel G:** *L. monocytogenes* + Sterilex + Trypsin. **Panel H:** *L. monocytogenes* + KC-610 + Trypsin

CHAPTER V

DISCUSSION

Investigators working with biofilms have used a variety of methods of assessing 'biofilms' including scraping biofilm material with swabs or staining biofilms with conventional crystal violet (CV) as a measure of biofilm quantity (Djordjevic, Wiedmann, & McLandsborough, 2002; Stepanovic, Vukovic, Hola, Di Bonaventura, Djukic, Cirkovic, & Ruzicka, 2007). These methods do not quantify the number of bacterial cells involved in biofilms because they do not quantitatively recover all the biofilm material and/or the bacterial cells may be clumped together by the protein and extracellular polysaccharide matrix (EPS) holding biofilm together. Also, CV staining does not bind specifically to bacterial cells (which may also be buried in biofilm, limiting diffusion of CV), but may bind also to the complex of protein/polysaccharide in biofilm which would be variable dependent on the mix or organisms producing them that are involved in the biofilm matrix.

Our lab has used a microplate (96-well) assay in combination with a plate washer (to remove planktonic/loose cells) and incorporating modified carboxyfluorescein that only fluoresces when it is hydrolyzed by the esterases in the cytoplasm of viable cells, method that is the basis of cell detection during flow cytometry (Hoefel, Grooby, Monis, Andrew

s, & Saint, 2003). This method was effectively used to screen the strains of *L. monocytogenes* isolated from 3 RTE meat processing plants and was able to differentiate between strongly- and weakly-adherent strains (Gamble & Muriana, 2007). The 96-well microplate format has been extensively used to study bacterial characteristics such as invasiveness and adherence (Nizet, Smith, Sullam, & Rubens, 1998). Moreover, microtiter plates have also been helpful for assessment of biofilm formation (Djordjevic, Wiedmann, & McLandsborough, 2002) and study of biofilm disinfection or removal (Pitts, Hamilton, Zelver, & Stewart, 2003). The prospect of being able to do various analyses in the wells of a single microplate makes this method swift, convenient, and cost-effective. The availability of microplate washer and plate reader systems further encouraged us to use 96-well plates and conduct adherence as well as lethality assays on them.

The original carboxyfluorescein assay used 5,6-carboxfluorescein diacetate (5,6-CFDA) whereby the colorless and non-fluorescing substrate readily diffuses into bacterial cells, gets hydrolyzed to a brilliant green fluorescing byproduct by cytoplasmic esterases, and the diacetate moiety prevents leakage from bacterial cells. It is also non-lethal to bacterial cells, so after a fluorescence assay, the adhered cells may be detached and used for enumeration by microbial plate counts. Hence, we pursued with screening two esterified fluorogenic substrates that can quantify fluorescence from live cells in biofilms. New modified versions have been developed, such as 5,6-carboxyfluorescein diacetate, succinimidyl ester (i.e., 5,6-CFDA, SE; also known as 5,6-CFSE) suggesting that the hydrolyzed fluorescent adduct could provide longer lasting fluorescence due to covalent binding to cytoplasmic proteins. Hence we evaluated both of these (5,6-CFDA and 5,6-

CFDA/SE) in order to use the better performing fluorescing substrate for the remainder of our biofilm assays.

Our results showed that 5,6-CFDA is a more suitable fluorescent substrate for assessing adherence in our microplate assay as used by Gamble and Muriana (2007). We obtained significantly different ($p < 0.05$) fluorescence signal values (i.e., RFU) from *Listeria* biofilms with 5,6-CFDA and 5,6-CFDA, SE dyes (Fig. 5) even though the subsequent detachment and enumeration of cells from wells treated with both dyes showed similar numbers (around 8 log CFU/ml). The superiority of 5,6-CFDA over 5,6-CFDA,SE in terms of indicating bacterial esterase activity correlating with bacterial numbers has been previously reported (Hoefel, Grooby, Monis, Andrews, & Saint, 2003). The bacterial esterase activity is essential to cleave/hydrolyze the substrate and give fluorescence. As argued by Hoefel et al. (2003), low activity of 5,6-CFDA,SE as fluorescent substrate may be due to mode of action of its specific succinimidyl ester (SE) group and also due to possible non-enzymatic cleavage of the substrate. These reasons might have caused low levels of RFU values with 5,6-CFDA,SE in our trials and feel confident that 5,6-CFDA is the best choice for use in our assays.

There are many studies explaining the ability of bacteria to attach to surfaces (Dourou, Beauchamp, Yoon, Geornaras, Belk, Smith, Nychas, & Sofos, 2011; Kushwaha & Muriana, 2009). Various factors such as culture concentration and age, pH, temperature, and time influence the attachment of bacteria (Fletcher, 1977; Garrett, Bhakoo, & Zhang, 2008). Numerous previous researches have shown that the adherence characteristics of bacterial cells differ among species and even within strains of same species (Barak, Whitehand, & Charkowski, 2002; Borucki, Peppin, White, Loge, & Call, 2003; D. H.

Meyer, Bunduki, Beliveau, & Donnelly, 1992). Our research also showed that there is significant difference ($p<0.05$) in levels of adherence among strongly and weakly adherent strains of *L. monocytogenes*, *E.coli* O157:H7 and *Salmonella* spp. The difference in adherence of *L. monocytogenes* 99-38 (strongly adherent) and *L. monocytogenes* CW35 (weakly adherent) obtained in this research confirmed the results shown by Tiong and Muriana (2016). Similarly, high level of attachment was also seen in one of the *E.coli* O157:H7 strains. Although the fluorescence microplate assay values for the *Salmonella* strains (even for the one categorized as strongly adherent) were relatively low compared to adherent strains of *L. monocytogenes* and *E. coli*, we selected the most adherent strain among the *Salmonella* to continue our microplate lethality assays with sanitizers on biofilms of strongly adherent strains from these 3 groups of pathogens (Fig. 10). These data suggest that *Salmonella* spp. may be weak at retention of 5,6-CFDA, or possibly that biofilms may exude a protective coating that limits 5,6-CFDA entry by diffusion. This proposition that bacterial species might have varied levels of fluorescence labeling or retention capability has been studied before (Drevets & Elliott, 1995). Another possible reason that can be argued for low RFU values with *Salmonella* is the incubation time of 15 minutes with the substrate, which may not be enough for the 5,6-CFDA substrate to enter the cells and get hydrolyzed to provide fluorescence.

The use of enzymes to remove cells adhered to surfaces is not a novel idea. This approach has been used for many years in studies with tissue culture of eukaryotic cells for cancer research, microbial virulence, and cellular mechanisms. The efficacy of proteolytic enzymes or proteases including proteinase K (Nguyen & Burrows, 2014), trypsin (Gilan & Sivan, 2013), and Bax protease (Gamble & Muriana, 2007), to remove

or detach the biofilm cells have been reported. Similar to their findings, the results we obtained also indicate that protease enzymes such as Bax protease, trypsin and pronase E are able to detach high numbers of cells, as high as 8-9 log CFU/ml) from *L. monocytogenes, Salmonella,* and *E. coli* biofilms from small well microplates (Fig. 12, 13 and 14). Although 'Bax' protease was effective, this was a proprietary and undisclosed protease supplied as part of the PCR assay for DuPont Bax PCR assays. Our interest was in finding an equally-effective and low-cost protease that could also be used in conjunction with sanitation regimens in food processing facilities. The ability of trypsin to detach cells was further visualized through SEM imaging which showed sparsely populated surfaces after treatment (Fig. 23, Panel E) and hence further confirmed our findings. Studies have shown that along with proteases other enzymes which degrade nucleic acids and polysaccharides are also able to disturb the cohesiveness of EPS matrix (Lequette, Boels, Clarisse, & Faille, 2010; Xavier, Picioreanu, Rani, van Loosdrecht, & Stewart, 2005). The ability of enzymes to hydrolyze protein-based bacterial 'appendages' such as flagella, pilli, fimbrae which may also be involved in attachment, or those that interfere with the EPS complex may allow attached cells to loosen up and be easily removed. This could likely be the reason we were able to get high detachment of cells with proteases such as trypsin.

The objective of our study was to optimize the microplate fluorescence adherence and lethality assay and then evaluate the efficacy of various commercial sanitizers on biofilms of foodborne pathogens. We evaluated 5 different sanitizers for their ability to inactivate bacterial biofilms and found varying results. BiQuat and 10-Chlor at 200 ppm each did not give as significant reduction in biofilm population as given by KC-610, Sterilex, and

Decon7 sanitizers even with comparatively longer treatment periods. BiQuat (200 ppm) was found to work best against *L. monocytogenes* 99-38 biofilms than *E.coli* O157:H7 and *S.* Montevideo FSIS051. The 2 hours of treatment showed complete elimination of *L. monocytogenes* 99-38 (>6 log CFU/ml reduction) but only a 1-log CFU/ml reduction of *E. coli* O157:H7 and *S.* Montevideo FSIS051 biofilms (Fig. 15). Except for 10-Chlor (10% sodium hypochlorite) at 200 ppm, all other sanitizers used were able to kill *L. monocytogenes* 99-38 biofilms within at least 30 minutes of treatment. Increasing the concentration of 10-Chlor to 1000 ppm however, gave complete inactivation of biofilms in 15 minutes of treatment (Fig. 17). A research study conducted on treatment of *L. monocytogenes*, *Pseudomonas fragi*, and *Staphylococcus xylosus* biofilms for 20 minutes with 10% sodium hypochlorite (200 ppm free chlorine), showed <0.5 log CFU/ml reduction of the organisms (Norwood & Gilmour, 2000).

Similarly, when we increased the concentration to 1000 ppm, the reduction was enhanced and 2 log CFU/ml reduction was achieved in 20 minutes. However, both these common sanitizers, BiQuat (QAC) and 10-Chlor (sodium hypochlorite) are only allowed to be used in concentration of <200 ppm on food contact surfaces, which doesn't seem to be effective in eliminating biofilms as per our findings. In order to be allowed for use at 1000 ppm, processors would have to first have a rinse treatment with water following sanitizer treatment in order to rinse residual sanitizer >200 ppm from food contact surfaces.

Three relatively new commercially available sanitizer formulas that we tested showed very effective results against biofilms. KC-610, Sterilex and Decon7 sanitizer solutions were all able to eliminate *L. monocytogenes* 99-38 biofilms within 5 minutes of

treatment, making it the most susceptible pathogen in our test. Similarly, S. Montevideo FSIS051 biofilm was the most resistant pathogen, as no other sanitizers except Decon7 (10%), were able to completely inactivate it. KC-610 formula (mixture of QAC and hydrogen peroxide with diacetin activator) showed a decrease in number of *L. monocytogenes* 99-38 and E. coli O157:H7 F4546 cells to the limit of detection (2 log CFU/ml) within 2.5 minutes of treatment and inactivation of *S.* Montevideo FSIS051 within 30 minutes. Thus we can observe from efficacy of KC-610 that mixing one or more disinfectant components can in fact enhance the sanitizer performance. We were also able to visualize the killing effect of KC-610, Sterilex and Decon7 sanitizers through SEM. The treatment with these sanitizers alone gave reduction in cells compared to untreated control (Figure 23, Panels B, C, and D). However, microbial enumeration from same (duplicate) treatments did not give any counts on plating as they were below the LOD. This suggests that the sanitizers can cause killing effect but may not remove the dead cells from biofilms. This case was further strongly supported when sanitizer treatment was subsequently followed with trypsin detachment. This combined treatment showed areas devoid of any bacterial biofilm (Fig. 23, Panels F, G and H) when observed under SEM and hence confirmed the detachment ability of enzymes to clean up surfaces from prior existing biofilms.

Hence, the common sanitizers such as QACs and sodium hypochlorites, being used in the food industries may not be effective in eliminating biofilms in food processing plants. However, the reductions achieved by use of new sanitizers which are based on combination of one or more components are encouraging and they definitely look like better alternatives to kill biofilms. Similarly, the results shown by detachment of

biofilm cells and its observation under SEM suggests the use of two steps; first, sanitizer application and second, enzyme treatment as the best option to kill as well as remove (dead) biofilms in food processing facilities.

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VITA

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Master of Science

Thesis: MICROPLATE LETHALITY ASSAY TO DETERMINE THE EFFICACY OF COMMERCIAL SANITIZERS FOR INACTIVATION OF *LISTERIA MONOCYTOGENES*, *ESCHERICHIA COLI* O157:H7, AND *SALMONELLA* IN BIOFILMS.

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