APPLICATION OF A NEXT GENERATION QUATERNARY AMMONIUM CHLORIDE SANITIZER AGAINST *Staphylococcus* AND *Pseudomonas* LABORATORY BIOFILMS AND NATURAL BIOFILMS FOUND ON WORKER'S BOOTS FROM A MEAT PROCESSING PLANT

By

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AGAINST *Staphylococcus* AND *Pseudomonas*

LABORATORY BIOFILMS AND NATURAL

BIOFILMS FOUND ON WORKER'S BOOTS FROM A

MEAT PROCESSING PLANT

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Title of Study: APPLICATION OF A NEXT GENERATION QUATERNARY AMMONIUM CHLORIDE SANITIZER AGAINST *Staphylococcus* AND *Pseudomonas* LABORATORY BIOFILMS AND NATURAL BIOFILMS FOUND ON WORKER'S BOOTS FROM A MEAT PROCESSING PLANT

Major Field: FOOD SCIENCE

Abstract: Foodborne pathogens are known to adhere strongly to surfaces and can form biofilms in food processing facilities whereby the potential to contaminate manufactured foods underscores the importance of sanitation, but all too often they are applied with little or no validation.

The objective of the study was to 1) confirm sanitizer (Decon7) effectiveness on biofilms of *Staphylococcus* and *Pseudomonas,* 2) validate sanitizer effectiveness on workers' boots from a slaughter floor environment, 3) identify biofilm bacteria from old boots in relation to previous sanitizer chemistry, and 4) evaluate enzymatic treatment to breakdown biofilms prior to sanitizer application.

A sanitizer that demonstrated superior effectiveness against *E. coli* O157:H7, *Salmonella* spp., and *L. monocytogenes* was applied at two concentrations against enhanced biofilms of strains of *Staphylococcus* spp. and *Pseudomonas* spp. (as required by EPA) in 96-wells microplates. Additionally, worker boots were swabbed with trypsin solution and then treated with the sanitizer spray solution. Bacteria isolated (before treatment) were identified by 16S rRNA PCR and DNA sequencing.

All treatments were carried out in triplicate replication and analyzed by RM-ANOVA using the Holm-Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$). The data show a significant difference between sanitizer treatment and control groups. There was a ~4-5 log reduction of bacterial strains (microplate assay) within the first 1 min of treatment and also greater > 3-log reduction in bacterial population from encrusted biofilms from workers' boots.

The new, next generation QAC (quad) sanitizers may be more effective than prior single/dual-QAC sanitizers and enzyme pre-treatment can facilitate biofilm sanitizer penetration on any food contact surface. Rotation of sanitizer chemistries may prevent selective retention of chemistry-tolerant microorganisms if they may occur.

Keywords: Biofilm, sanitizer, *L. monocytogenes, Salmonella, E. coli* O157:H7, *Pseudomonas, Staphylococcus*, microplate assay

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CHAPTER I

INTRODUCTION

Microorganisms can establish a persistent presence in food processing facilities by adhering to surfaces and then creating a biofilm. Biofilms primarily consist of microbial cells and their exopolysaccharides (EPS), which is the primary matrix material (i.e., the 'glue') that holds them together. It may be comprised of protein, nucleic acids, lipids, polysaccharides, glycoproteins, glycolipids, and extracellular DNA. Biofilms generally require a) a surface to attach to, b) nutrients, and c) water. These are all present in food processing facilities. Bacteria can more readily bind to surfaces that are rough, scratched, cracked, corroded, and/or already have an attached layer of organic debris. It occurs as a series of events that includes attachment of cells to the surface using electrostatic/hydrophilic interactions, followed by EPS production that creates irreversible attachment (the 'glue'), the formation of microcolonies, and maturation into a complex biofilm. Dispersal of cells from the biofilm by sloughing off of pieces or discharge of growing cells can lead to distribution and spread of the organisms in the food processing environment.

The EPA registers different sanitizers for food contact surfaces or non-food contact surfaces. A registered sanitizer must show a reduction in test organisms; 99.9% for non-food contact sanitizers and 99.9999% for food contact sanitization (contact time for efficacy testing < 10 minutes). To secure a disinfectant claim, the sanitizer should show an effectiveness against the *Staphylococcus aureus* (or *Salmonella choleraesuis*) to be registered as a broad-spectrum disinfectant. In addition to that, a hospital disinfectant claim also requires superior effectiveness against *Pseudomonas aeruginosa.*

Contingent upon the literature about the robust biofilms that could be developed by *Pseudomonas aeruginosa* and *Staphylococcus aureus,* we evaluated the sanitizer effectiveness against these organisms. In our research, we tried to simulate the natural environment to create robust biofilms by repeatedly washing and re-supplying nutrient medium to microplate wells for seven consecutive days. Microplate biofilm assay is one technique to achieve a high bacterial growth in a biofilm. The method we employed helped to achieve high bacterial growth in biofilms and was optimized to form extended biofilms of strongly adherent strains of *L. monocytogenes, Salmonella* and *E. coli* O157:H7. Proteolytic enzymes like trypsin has been found to faciltate detachment assay to recover viable cells from those extended biofilms, either before or after sanitizer treatment (Aryal, Pranatharthiharan, & Muriana, 2019). Compared to other sanitizers like Bi-Quat (Simple QAC sanitizer), 10-Chlor (Hypochlorite based sanitizer), Sterilex solution (New generation QAC) and KC-610 (Peroxy acetic acid based sanitizer) used against *Listeria monocytogenes, E. coli* O157:H7, and *Salmonella* Montevideo, Decon7 was the most effective and had the greatest log reduction in the shortest period of application (Aryal & Muriana, 2019).

This work is an extension of prior work done in our lab to characterize the microplate biofilm assay. In this study, we examined the effect of Decon7 on strains of *Pseudomonas* that has been a problem in liquid processing egg facilities and strains of *Staphylococcus* to facilitate claims that Decon7 works against biofilms. Additional studies examined the practical application of Decon7 in the FAPC Meat Processing Pilot Plant and its effect on biofilms (generic) that developed with repeated-use of Bi-Quat (not highly effective; selects for alkaliphilic organisms). Bacteria isolated from workers' boots (before treatment) were identified in relation to prior sanitizer chemistry by 16S rRNA PCR and DNA sequencing and a comparison test was carried out between Decon7 and Bi-Quat to see the relative antimicrobial activity against the strains isolated from the FAPC processing plant.

CHAPTER II

REVIEW OF LITERATURE

Biofilm structure and architecture

Microorganisms support themselves by adhering to various surfaces, thus creating a biofilm (Donlan, 2002). In the natural environment, biofilms are generally formed by a mixture of different microorganisms that can be intra- or interspecies (Sutherland, 2001). Biofilms primarily consist of microbial cells and their extracellular polysaccharide (EPS), which is the primary matrix material. The different microorganisms may have different EPS that can vary in chemical and physical properties. It is generally comprise of proteins, nucleic acids, lipids, polysaccharides, proteins, glycoproteins, glycolipids, and extracellular DNA (e-DNA) but polysaccharides are its key material (Flemming, Wingender, Griegbe, & Mayer, 2000).

Exopolysaccharides (EPS)

Microorganisms adhere and incorporate themselves onto the surfaces through exopolysaccharides. The cohesion between the microbes helps in different interactions, including communications between the cells and forming a matrix that creates synergism

(Flemming & Wingender, 2010). Besides adherence and cohesion of bacterial biofilms, EPS acts as a protective barrier and performs several functions such as water retention, nutrient source, enzymatic activity, and exchange of genetic information

Biofilm formation

Biofilm is formed in a series of events that include attachment of the cells to the surface, followed by EPS production that creates irreversible attachment, the formation of microcolonies, maturation, and dispersal of cells from the biofilm (Stoodley, Sauer, Davies, & Costerton, 2002). First, a bacterium adheres to a surface that along with nutrients, moisture, and organic molecules to form a bacterial biofilm. These constituents on the surface can facilitate the attachment of bacteria. The initial attachment is facilitated by the van der Walls interaction that later forms a receptor-mediated interaction through the help of pilli, flagella, exopolysaccharides, and lipopolysaccharides (Donlan, 2002). The bacterial cells, once attached to a surface, begin multiplying and form several microcolonies. These microcolonies further mature and coalesce together and develop water channels within a matrix. Once the supply of nutrients gets scarce, the cells within that biofilm detach and disperse and colonize the neighboring surfaces (G. O'Toole, Kaplan, & Kolter, 2000).

Biofilm architecture

Most biofilms are flat and compact while others have more complex structures varying from patchy clumps, pillars-like, and mushroom-shaped. (Shrout, Tolker-Nielsen, Givskov, & Parsek, 2011). There are various factors that play a role in the development of typical structures of a biofilm; the characteristics of the attached surfaces, the number of

initial attachment of the cells, the particular organism/mix colonies forming the biofilm, the transcription factors as well as the physical and chemical properties of the surrounding environment (Rudge, Steiner, Phillips, & Haseloff, 2012). Within the biofilm structure, different microenvironments are developed. The bacterial exopolysaccharides limits the transfer of nutrients and chemicals into the biofilm (Stewart & Franklin, 2008). The only possible way of transfer of nutrients and chemicals is through a diffusion mechanism (Stewart, 2003). Thus, the cells on the surface of the biofilm receives fresh medium and nutrients more readily as compared to the cells deeper inside the biofilm. The cells deeper inside a biofilm may adapt to a new microenvironment by altering their metabolism and gene expression thereby resulting in heterogeneity of cells in the biofilm (Sauer, Camper, Ehrlich, Costerton, & Davies, 2002; Stewart & Franklin, 2008).

Quorum sensing and gene regulation

Quorum sensing is a cell to cell communication mechanism by which many species of bacteria within a biofilm communicate (Ng & Bassler, 2009; Waters & Bassler, 2005). Based on their population density, quorum sensing helps coordinate gene expression with the presence of other cells. During this process, the receptors of new bacteria receive the signaling molecules, and may initiate the transcription of genes within and between the bacteria (Miller & Bassler, 2001). It allows communication between microorganism's species regarding nutrient shortages and environmental conditions such as the presence of disinfectants and antibiotics. It has been found that bacteria use quorum sensing to modulate or produce virulence factors (Rutherford & Bassler, 2012). Quorum sensing effects gene expression within the bacterial community based on the cell density and species complexity. Gene expression is favored at low cell density when a bacterium has

to show its individual behavior and is favored at high cell density when the bacterium has to show a group response or social behavior (Ng & Bassler, 2009). Bacteria releases their chemical signal molecules known as autoinducers intracellularly which alter gene expression and controls the behavior of the population. With increases in population, the extracellular concentration of autoinducers also increases and when it reaches a threshold detection level, the receptors binds with the autoinducers and activate signal transduction. This ultimately causes changes in gene expression and helps bacterial cells in a biofilm to function inclusively (Ng & Bassler, 2009; Waters & Bassler, 2005).

Quorum sensing in Gram-negative bacteria

Most of the Gram-negative bacteria communicate with acyl-homoserine lactone (AHL) signals through LuxIR-type proteins (Manefield & Turner, 2002). *Vibrio fischeri* is an example of a Gram-negative bacterium that induce expression of genes for bioluminescence at high cell density. LuxI and LuxR are two proteins that produces AHL autoinducer *N*-3-(oxo-hexanoyl)-homoserine lactone (Engebrecht & Silverman, 1984) and cytoplasmic autoinducer receptor (Engebrecht, Nealson, & Silverman, 1983) respectively. Increase in cell density increases the production of AHL and at a threshold detection limit, it binds with LuxR and activates the Lux operon that expresses bioluminescence and also triggers the production of *luxI* gene (Stevens, Dolan, & Greenberg, 1994). LuxR type proteins activating *luxI* type genes are commonly found in AHL-QS systems. *Pseudomonas* aeruginosa have a LasI/LasR-RhlI/RhlR system which controls the gene expression for virulence factor and biofilm formation (Passador, Cook, Gambello, Rust, & Iglewski, 1993; Pearson, Passador, Iglewski, & Greenberg, 1995). Likewise, the TraI/TraR system of *Agrobacterium tumefaciens* regulate transfer of plasmid to host (Fuqua &

Winans, 1994) and the EsaI/EsaR system in *Pantoea stewartii* controls EPS secretion, surface attachment, adherence, and colonization (Von Bodman & Farrand, 1995). Thus, the AHL type of quorum sensing is intraspecific.

Quorum sensing in Gram-positive bacteria

Unlike the AHL type QS inducer in Gram-negative bacteria that is cytoplasmic, the QS inducer in Gram-positive bacteria is membrane-bound and has oligopeptides as signals that is mediated through a phosphorylation cascade and histidine kinases as receptors (Waters & Bassler, 2005). The secretion of oligopeptide autoinducers increases with an increase in cell density. *Staphylococcus aureus* is an example of a Gram-positive bacteria that has a two-component response regulatory system known as Agr quorum sensing system. The autoinducer is called the auto-inducing peptide (AIP) which is encoded by the *agrD* gene. AgrC and AgrA are the kinase response regulatory pair that detects extracellular AIP and causes signal transduction respectively while AgrB is a protein that adds the thiolactone ring to the AIPs (Kong, Vuong, & Otto, 2006). These factors play an important role in virulence of the bacterium. It expresses protein factors that facilitate attachment and multiplication at low cell density while at high cell density it suppresses these factors and promotes the secretion of toxins and proteases that helps in dispersal of the bacterium (Lyon & Novick, 2004). Like the AgrC/AgrA system in *Staphylococcus aureus* (Ji, Beavis, & Novick, 1995), the ComD/ComE system of *Streptococcus pneumoniae* and the ComP/ComA system of *Bacillus subtilis* (Magnuson, Solomon, & Grossman, 1994) controls competency.

Biofilm of *Pseudomonas aeruginosa*

The stability of the biofilm structure is regulated by its three polysaccharides; alignate, Pel and Psl that either forms mucoid or non-mucoid strains (Ryder, Byrd, & Wozniak, 2007). Alignate is the primary contributor to the structural stability and protection of the biofilms. It helps in the retention of water and nutrients in the biofilm and is composed of D-mannuronic acid and L-guluronic acid in a linear and unbranched form (Ma et al., 2009; Ma et al., 2012). Besides these, extracellular appendages like flagella, type IV pilli and fimbriae also play an important role in cell to surface attachment and micro colonies formation (G. A. O'Toole & Kolter, 1998). Cell-to-cell signaling primarily control the maturation of *P. aeruginosa* biofilms. In many Gram-negative bacteria like *P. aeruginosa,* QS system depends on acylated homoserine lactones. These AHLs are produced at higher levels and acts as ligands for transcriptional regulators when the bacterium cell density is high (Davies et al., 1998). The transcriptional regulators LasR and RhlR are activated by the synthesis of two AHLs, N-3-oxododecanoyl homoserine lactone (3OC12-HSL) and N-butyryl-homoserine lactone (C4-HSL). The activation of these regulators initiates gene regulation for the production of proteases, elastases, toxins, and hemolysins thus initiating the virulence of the bacterium (Rumbaugh, Griswold, & Hamood, 2000). During an early development of the biofilm formed by *P. aeruginosa*, the presence of eDNA plays a crucial role as a cell-to-cell interconnecting compound (Allesen-Holm et al., 2006; Flemming & Wingender, 2010; Whitchurch et al., 2002). It also produces an extracellular deoxyribonuclease (eDNA) that helps in utilizing eDNA as nutrient source to the biofilm during initial development and when the bacterium is deprived of any essential sustenance for survival (Finkel & Kolter, 2001; Mulcahy, Charron-Mazenod, & Lewenza, 2010).

Biofilm of *Staphylococcus aureus*

Though it has been found that QS systems have been beneficial for *P. aeruginosa* to develop a robust biofilm, the same case is not for *S. aureus* where an active QS impedes the attachment and development of a biofilm (Beenken, Blevins, & Smeltzer, 2003; Vuong, Saenz, Götz, & Otto, 2000). Staphylococci have the accessory gene regulator (Agr) locus as the only quorum sensing system that produces communication molecule called AIP. Recent studies have shown that the presence of an active quorum-sensing impedes attachment and development of a biofilm as the Agr system has been found to be active in cells detaching from a biofilm (Boles & Horswill, 2008). There is the involvement of an Agr quorum-sensing system in biofilm detachment. Bacteria dispersing from biofilms may display high levels of Agr activity, while cells in a biofilm have predominantly repressed Agr systems. These results show that Agr deficient *S. aureus* strains form more robust biofilms (Yarwood, Bartels, Volper, & Greenberg, 2004).

Mixed biofilm

Biofilms in a natural environment usually consists of multiple species living together as a community. Mixed species biofilms are a more robust and persistent form than mono-species and the organisms on mixed biofilm generally develops metabolic cooperation and co-aggregation. Though, there can be antagonistic effects like competition for nutrients and space among the species in a biofilm, organisms can develop synergistic relationships (Elias & Banin, 2012). An AI-2 system and AHL is mostly found in mixed biofilms that mediates cell-cell communication. The biofilm growth of *Salmonella agona* was higher (threefold increase) in the presence of *Staphylococcus* and *Pseudomonas* in drip-flow

reactors (Habimana, Meyrand, Meylheuc, Kulakauskas, & Briandet, 2009). Likewise, the biofilm of *S.* Typhimurium grew better in the presence of other strains including *Paenibacillus, Bacillus*, and *Enterococcus* (Schaefer, Brözel, & Venter, 2013). *P. aeruginosa* was found to facilitate microcolonies of *S. aureus* when grown together. Both of these organisms shared eDNA in the EPS matrix that facilitated the interspecies interaction (Yang et al., 2011). Lee et al., in his study tested three different microorganism, *P. aeruginosa* PAO1, *K. pneumoniae* KP-1 and *P. protegens* Pf-5 against tobramycin where Pf-5 was only the resistant species against tobramycin while the other two were sensitive. However, in a mixed biofilm all of these three species acquired protection against tobramycin and their bio-volume per unit base area was higher than when grown individually (Lee et al., 2014).

Antimicrobial resistance

The antibiotic resistance mechanism of biofilm bacteria varies among different conditions, and it occurs through direct inactivation of the active molecule, altering the body's sensitivity to the target of the action, and reducing drug concentrations and efflux systems. It is believed that biofilms may have some intrinsic mechanisms responsible for antibiotic resistance (Nickel & Costerton, 1993). It is found that several mechanisms like limited diffusion, enzyme neutralization, heterogeneous functions, slow growth rate, persistent cells, and adaptive mechanisms such as efflux pump and membrane alteration as critical factors in high resistance nature of biofilms (Hogan & Kolter, 2002). Exopolysaccharide acts as a physical barrier for the diffusion or penetration of antibiotics to deeper layers of biofilm. Antibiotics interact with this matrix and result in their slow movement to the interior of the biofilm. System proteins and lysosomes that have high molecular weight are

hindered from passing through the EPS. Thus, bacteria are quickly attacked by the immune system that does not produce polysaccharide. It is reported that *P. aeruginosa* has anionic EPS called alginate exopolysaccharide so, fluoroquinolones and aminoglycosides do not penetrate readily (Mah & O'Toole, 2001).

Other mechanisms, such as slow diffusion, have been assumed to be involved in biofilm resistance to antibiotics. Slow diffusion permits plenty of time to establish a protective response to stress (Mah & O'Toole, 2001). The cells that are present at the bottom layer of a biofilm are limited to oxygen and nutrients but their position enhances the resistance towards any antimicrobials and contribute to their persistence in biofilms (Dufour, Leung, & Lévesque, 2010). Bacteria in a biofilm also express stress-responsive genes under heat, cold, pH, osmolarity and different environmental stressors. The formation of persister cells when certain specialized cells enter dormancy state, allows the cells to survive in stress conditions and protects themselves from the antimicrobial treatment (Lewis, 2010). Likewise, the alarmones and pheromones produced in stress condition within a biofilm community aids in antimicrobial tolerance (Fux, Costerton, Stewart, & Stoodley, 2005).

Biofilms in food processing environments

Dairy industry

Most bacteria are associated with the dairy industry's contact surfaces in milk storage and dairy processing operations, and numerous other industrial systems, are often present only in the raw material (Marchand et al., 2012). There is a potential source of contamination through the attachment of bacteria and subsequent development of biofilms on finished products that may shorten the shelf life or facilitate disease transmission (Hood & Zottola,

1995). Biofilm accumulates organic matter on a metal surface under suitable conditions and various environmental and physiological factors trigger a planktonic cell to form a biofilm. These factors are listed as quorum sensing, nutrient availability, and cellular stress (Sauer, Rickard, & Davies, 2007). Biofilm communities also possess the selective pressure required for programmed cell death which eliminates damaged individuals from the population (Bayles, 2007). Bayles further demonstrated that programmed cell death maintains healthy individuals by utilizing the dead cells' available nutrients. It also reduces competition among the population and maintains their spatial orientation within the biofilm. Besides, the released genomic DNA from the damaged cells maintains the stability of biofilm's overall structure (Bayles, 2007).

At first, there occurs the formation of a conditioning layer followed by bacterial adhesion, bacterial growth, and biofilm expansion (Kumar & Anand, 1998). This conditioning layer mainly consists of organic milk components (Mittelman, 1998). Once established in such a processing plant, biofilms accelerate corrosion and material deterioration (Storgards, Simola, Sjöberg, & Wirtanen, 1999a). The presence of dead ends, corners, cracks, crevices, gaskets, valves, and joints in the dairy industry are all possible points for biofilm formation (Storgards, Simola, Sjöberg, & Wirtanen, 1999b). The biofilm structures formed mainly depend on the intrinsic and extrinsic factors such as bacterial species, temperature, flow conditions, pH, and nutrients (McLandsborough, Rodriguez, Pérez-Conesa, & Weiss, 2006). Likewise, at higher temperatures (37ºC), the cell surface hydrophobicity increases and results in the subsequent bacterial attachment compared with 4, 12, and 22 °C (Cappello & Guglielmino, 2006). Likewise, several groups have reported that in milk processing environments, bacteria can easily attach to rubber and stainless steel

(Czechowski, 1990). De Jong (1997) showed that heat processes above 65 °C denature and aggregate whey proteins in milk, leading to a faster adherence of bacteria to stainless steel. Flint and his team (Flint, Palmer, Bloemen, Brooks, & Crawford, 2001) discovered that vegetative cells and spores of *G. stearothermophilus* are attracted to fouled surfaces by 10–100 times more than clean stainless steel. Milk spoilers and pathogens from the dairy farm and processing environment and rinsing water can contaminate the milking equipment (Oliver, Jayarao, & Almeida, 2005). Microorganisms like *Pseudomonas*, *Aeromonas* and *Legionella* from the rinsing water can develop biofilms that could be problematic in their removal (Momba, Kfir, Venter, & Cloete, 2000). They can also act as a source to harbor other microorganisms, increasing the probability of pathogen survival and spreading in milk processing (Lomander, Schreuders, Russek-Cohen, & Ali, 2004). (Teixeira, Lopes, Azeredo, Oliveira, & Vieira, 2005) found that the short rubber milking tube in automatic milking machines is more prone to biofilm formation. Prior to heat processing, *Pseudomonas spp*. are more likely to generate multispecies biofilms on the walls of milk cooling tanks or pipelines.

Sometimes there may occur the development of a single-species biofilm due to pasteurization. During this process, heat-sensitive species are killed, leaving only the heat resistant species such as *Streptococcus bovis* or *Streptococcus thermophiles* (Bouman et al., 1982). *Pseudomonas* biofilms not only pose a risk of being a severe contaminant to milk batches but also may attract or harbor other spoilage or pathogenic bacteria. In this concern, (Simoes et al., 2009) illustrated that dual biofilms of *P. fluorescens* and *B. cereus* were about five times more metabolically active than *P. fluorescens* monospecies biofilms. Additionally, they conferred that the biofilm of *P. fluorescens* was more tolerant

to antimicrobials than *B. cereus* in single-species biofilms in terms of viability.

Poultry industry

In the poultry industry, the major contaminants in chickens are the presence of bacteria such as *Salmonella* and *Campylobacter*. There can be a rising level of infection due to unsanitary hygiene measures and low biosecurity standards. However, in addition to these environmental issues, the presence of biofilms is always a concern (Hanning, Jarquin, & Slavik, 2008). The minimum supply of water within the farm can lead to the formation and development of a biofilm. In a farm, drinking water fountains are more prone to bacterial attachment as these fountains have more significant biomass portions covered with rubber (Pometto III & Demirci, 2015). Ogden et al. (2007) added that one of the most common contamination sources are the biofilms formed inside the supply water pipe. Here, various microorganisms proliferate inside the pipe and continuously release planktonic cells into the water. Coolers and ventilation systems can also favor the microbial aggregation, especially when there is a preexisting biofilm (Pometto III & Demirci, 2015). Due to ample moisture during chicken processing steps, higher biofilm formation rates are predominantly found in plastic curtains, mats, tanks, chiller, and stainless steel tools (Srey, Jahid, & Ha, 2013). Jang et al. (2007) mentioned the presence of Campylobacter in the chicken's skin as another form of adaptation of the microorganism. These bacteria were able to fix into deep crevices of the skin and feather follicle of the bird where they received an ideal condition to adhere, colonize, form biofilms, and remain protected in the carcass, even at low temperatures.

Meat industry

Serious hygienic problems and economic losses due to food spoilage from the

contamination of biofilms formed by pathogenic and spoilage bacteria have occurred in the meat industry (Jessen & Lammert, 2003). Meat production and processing industries are more concerned with the ability of bacteria to attach to abiotic surfaces and form biofilms (Chmielewski & Frank, 2003). There are suitable conditions for bacteria in the meat industry to be attached to food contact surfaces like conveyor belts, tables, knives, and form robust biofilms. Once a biofilm develops, eradicating them with standard cleaning and disinfection procedures may not be fully effective (Joseph, Otta, Karunasagar, & Karunasagar, 2001).

Cross-contamination is an important factor for sporadic and epidemic foodborne illness and is a significant concern for producers and consumers when a cell detaches from the biofilms during the passage of food through contaminated surfaces (Reij, Den Aantrekker, & Force, 2004). Moreover, processing equipment is regarded as an essential carrier for cross-contamination throughout the meat chain (Gounadaki, Skandamis, Drosinos, & Nychas, 2008). Similarly, foodborne pathogens transferred from the equipment to non-inoculated meats have also been investigated (Papadopoulou et al., 2012). During ham slicing, the transfer of *S. aureus* and *E. coli* O157: H7 was reported (Pérez-Rodríguez et al., 2007)**.** The cross-contamination between processing equipment and deli meats of attached *L. monocytogenes* cells was also investigated where they observed 1, 2, and 3 log CFU per blade inoculation with low and random transfer results (Lin et al., 2006). In the meat processing industry, carcasses are frequently spray washed and rapidly chilled before transport and storage, resulting in psychrotrophs and psychrophiles being favored (Chamberlain & Johal, 1988). When studying adhesion properties of bacteria associated with meat handling, they are generally concentrated on the surface of meat surfaces (Butler,

Stewart, Vanderzant, Carpenter, & Smith, 1979). In addition, when freshly exposed meat surfaces are glided mechanically over the contact surface, there can be a possibility of significant cross-contamination.

Fish processing industry

As fish processing operations produce high nitrogenous wastes with carbon and phosphorous contents, these industries often face problems of waste handling and disposal (Chowdhury, Viraraghavan, & Srinivasan, 2010). Furthermore, aquaculture ponds are being fertilized with phosphorous and nitrogen; it serves as an important source of nutrients for biofilms. The available nutrients help in faster growth and formation of EPS (Pandey, Bharti, & Kumar, 2014). It is showed bacterial biofilms may colonize in fish tissues, including mollusks and crustaceans. Biofilm formation can occur on the surfaces of materials used, such as rubber, PVC, glass, and stainless steel demonstrated that during the harvesting of seafood, the baskets primarily made up of metal/plastic could trap particulate matter, and bacteria can attach to the harvesting material, leading to possible biofilm formation. In addition, as fish are tightly packed during transport, the contamination from the fish surface can transfer to the transport material (Rajkowski, 2009).

Measures to control biofilms in food industry

In any food industry, there are several control strategies against biofilms that ranges from physical, chemical and biological treatment such as use of plant extracts, disinfectants, sanitizers, bacteriophages, nanotechnology, bacteriocin, enzymes and so-forth (Sadekuzzaman, Yang, Mizan, & Ha, 2015). High temperatures are also used as a sanitizing treatment (Graham-Rack & Binsted, 1973).

Use of chemical sanitizers

Chemical sanitizers or disinfectants are used in removing the biofilms and they can include halogens, chlorine, iodine, quaternary ammonium compounds and various acids (Troller, 1983).. While the choice of sanitizer to be used depends on several factors and varies with application. Sanitizers shows its best performance when the organic compounds are removed from the surface material. Detergents helps in dissolving the organic matter and EPS matrix within a biofilm, which creates an open access for disinfectants/sanitizers to act on the exposed bacterial cells (Simões, Simões, Machado, Pereira, & Vieira, 2006). The chlorine sanitizers lack such penetrating ability and they would mostly react with the organic material present on the surface that is to be treated. Talking about iodine sanitizers, they are usually elemental iodine combined with wetting agents and buffered to a low pH. Thus, they have some penetrating ability but not a complete effect. Like iodine sanitizers, quaternary ammonium compounds have many configurations and usually are combined with a wetting agent so they do possess some penetrating ability. Acid sanitizers are composed of acid, anionic surfactants, nonionic surfactants and as well may lack detergency (Cords, 1983). Again, not all of these sanitizers accomplish complete removal of biofilm. *P. fragi, Salmonella montevideo* and *Bacillus cereus* biofilms treated with sodium hypochlorite showed that the treatment with sodium hypochlorite followed by water rinse was not sufficient to remove the attached organisms (Schwach & Zottola, 1984). Evaluation of nine disinfectants with four different species of *Listeria* showed that the efficacy of some chemicals used was influenced by the presence of organic matter. However, the four species of *Listeria* were not particularly resistant to the disinfectants (Van de Weyer, Devleeschouwer, & Dony, 1993). The effect of two different clean in place

(CIP) cleaning systems on stainless steel in a pilot sized milk transfer system containing the biofilms of *P. fragi* showed proper concentrations of sanitizers, temperature and flow rate were able to remove adherent bacteria and any remaining microorganisms in the biofilm (Stone & Zottola, 1985). Likewise, Czechowski & Banner (1992) investigated the efficacy of sanitizers on removal of biofilms, reducing the viability of bacteria from stainless steel, Teflon, and Buna-n gaskets in pipeline systems. Their results showed that when chlorinated alkaline detergents are combined with sanitizers, it would reduce the viable population to greater than 98 percent. Milledge & Jowitt (1980) showed, with proper chemical concentration, length of contact time, temperature and pH, it would be more important than the type and finish of stainless steel or other food contact surfaces.

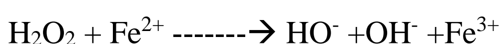
Quaternary ammonium chloride (QAC)

These are the amphoteric surfactants that has broad spectrum antimicrobial activity (McBain, Ledder, Moore, Catrenich, & Gilbert, 2004). The positively charged quaternary nitrogen and hydrophilic head of acidic phospholipids act upon the cytoplasmic and membrane lipid bilayers (Gilbert & Al-taae, 1985) while the hydrophobic tail solubilizes the hydrophobic membrane and lyse the cell (Salton, 1968). The mode of action of QAC follows a series of steps; QAC is adsorbed into the bacterial cell wall and reacts with lipid and protein present in the cytoplasmic membrane, which results in membrane disorganization and leakage of intracellular materials. The proteins and nucleic acids gets degraded and the cell lyses (McDonnell, 2020). Because QAC has been found to have low toxicity, many food industries use them as a sanitizer. But it has been observed that the use of QAC developed resistance in *Listeria* (Holah, Taylor, Dawson, & Hall, 2002) still these disinfectants are found to be effective against foodborne pathogens like *Listeria* (Kastbjerg

& Gram, 2012). The use of QAC at 200 ppm showed 99.99% reduction in *E.coli* O157: H7 and *Salmonella* (TAPP III, Gragg, Brooks, Miller, & Brashears, 2013).

Hydrogen peroxide (H_2O_2)

It is an environment friendly, non-toxic (easily degradable to water and oxygen), broad-spectrum disinfectant. But in presence of iron salts, it forms highly toxic hydroxyl radical through oxidization that is believed to act as a bactericide (Haber & Weiss, 1934).



$H_2O_2 + Fe^{2+}$ -------→ $HO^{-} + OH^{-} + Fe^{3+}$

The hydroxyl free radical degenerates the essential cell components like protein, lipids and DNA by attacking on the sulfhydryl groups and double bonds (Seymour Stanton Block, 2001). The use of hydrogen peroxide has been evident since a long time in food industries (Ukuku, 2004). When a bacterial suspension of *Listeria monocytogenes* on stainless coupon was exposed to hydrogen peroxide mist, a >5 log reduction of the organism was observed (Møretrø, Fanebust, Fagerlund, & Langsrud, 2019). Besides generating hydroxyl radical, H_2O_2 activates the lactoperoxidase-thiocyanate-H_2O_2 (LP) system that has been reported as an inhibitor for a wide range of Gram-negative (Björck, Rosen, Marshall, & Reiter, 1975) and Gram-positive bacteria (Björck et al., 1975; Oram & Reiter, 1966). In this system, H_2O_2 oxidizes the thiocyanate ion producing hypothiocyanate that has bacteriostatic activity.

Enzyme detachment of biofilm

The dead biofilms after the effect of antimicrobials may foster the reattachment of cells and create a new biofilm thus enzymes like proteases, glycosidases and deoxyribonucleases helps in detachment of cells from those biofilms. These enzymes degrade the extracellular

polymeric matrix of the biofilms (Kaplan, 2014). The detachment of cells from biofilms is dependent on the chemical composition. An enzyme dispersin B was shown to rapidly remove the biofilms of *S. epidermis* from catheters (Kaplan et al., 2004). A surfactant with the use of proteolytic enzyme and carbohydrolase was recommended for efficient biofilm removal (Johansen, Falholt, & Gram, 1997). Pancreatin enzyme having proteolytic, amylase and lipase activities also helps in removal of biofilm (Marion et al., 2005). Enzymes like Bax protease, pronase E, lipase, cellulase and trypsin were used for detaching cells from biofilms (Aryal et al., 2019). The combination of proteases and polysaccharide hydrolyzing enzymes showed effective breakdown of exopolysaccharides in a biofilm (Meyer, 2003). The environmental friendly and non-toxic nature of enzymes have been one major factor for using them in biofilm removal (Srey et al., 2013). However, the specificity in enzymes mode of action, cost compared to that of lower priced chemicals and lower commercial accessibility has limited their use (Simoes, Simões, & Vieira, 2010).

EPA validation of sanitizers

The Environmental Protection Agency regulate the use of pesticides/sanitizer- chemical composition, labeling and packaging. EPA registers different sanitizers: for non-food contact surfaces and food-contact surfaces. For a sanitizer to be registered, it must show a reduction in test organisms- 99.9% for non-food contact sanitizers and 99.9999% for food contact sanitization (contact time <10 minutes). To secure a disinfectant claim, the sanitizer should show an effectiveness against the *Staphylococcus aureus* or *Salmonella choleraesuis* while both, to be registered as a broad-spectrum disinfectant. In addition to that, a hospital disinfectant claim also requires a superior effectiveness against *Pseudomonas aeruginosa* (Sanders, 2003).

CHAPTER III

MATERIALS AND METHODS

Bacterial strains, storage and growth conditions

Cultures were from Dr. Muriana's culture collection, and included *Pseudomonas aeruginosa* 1 and *Pseudomonas aeruginosa* 2 that were problem isolates from commercial liquid egg pasteurization facilities as well as *Staphylococcus aureus* PMM 174C1, *Staphylococcus aureus* PMM 169C8 and *Staphylococcus equorum* PMM 854HS-7. These cultures were grown in tryptic soy broth (TSB, Bacto, BD, NJ, USA) at 30°C overnight and centrifuged (6000 × g, 5 °C) for 10 minutes. The supernatant liquid was discarded and freezing storage solution (sterile TSB + 10% glycerol) was transferred into the pellet. The pellet was vortexed to suspend the cells and 4 mL of each cell suspension was transferred into vial and stored at -80ºC. Frozen cultures were revived by thawing and transfer of 100 µL to 9 ml TSB, incubated overnight at 30ºC, and sub-cultured twice before use. All assays were performed in triplicate replication; separate cultures were grown for each replication, which were performed as independent experiments. Ten-fold serial dilutions were performed with buffered peptone water (BPW) and surface plated (100 µl, 0.1%) on tryptic soy agar, in duplicate. TSA plates were then incubated at 30ºC for 2 days before enumeration.

Growth of enhanced biofilms in microplates

Cultures of *Staphylococcus* and *Pseudomonas* were grown overnight at 30ºC (~9 log CFU/mL) and was then diluted to ~4 log CFU/mL in TSB for initial distribution into microplates. Falcon 96-well, clear, flat-bottom microplates (Cat# 351172, Corning, NY, USA) were used to create biofilms. Prior to using the microplates to create biofilms, we examined both treated plates and untreated plates for ability to generate robust biofilms. A 200-µl aliquot of the culture was allocated into microplates which were then sealed with parafilm to avoid evaporation and incubated at 30°C for 24 hours.

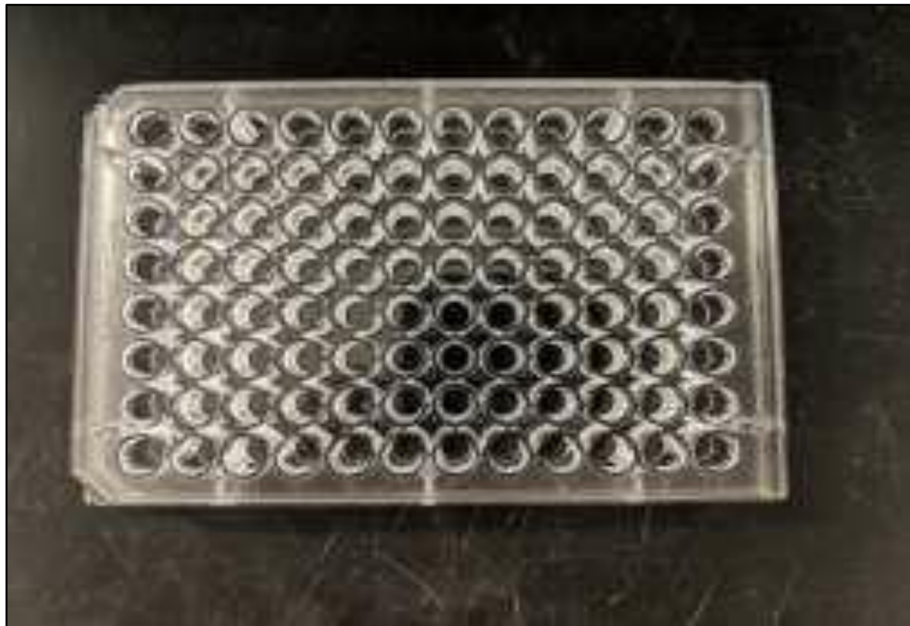


Figure 1. Microplate (96-well) used for creating biofilms.

After 24 hours, microplates were washed three times with sterile tris buffer (pH 7.4, 0.05 M) in a Biotek ELx405 Magna plate washer (Ipswich, Suffolk, UK) and 200 µl of fresh sterile TSB containing select bacterial strains was added into the wells. Microplates were then sealed with parafilm and incubated for 24 hours at 30°C. This cycle of growing, washing, and media renewal was repeated daily for 7 days to develop robust biofilms.

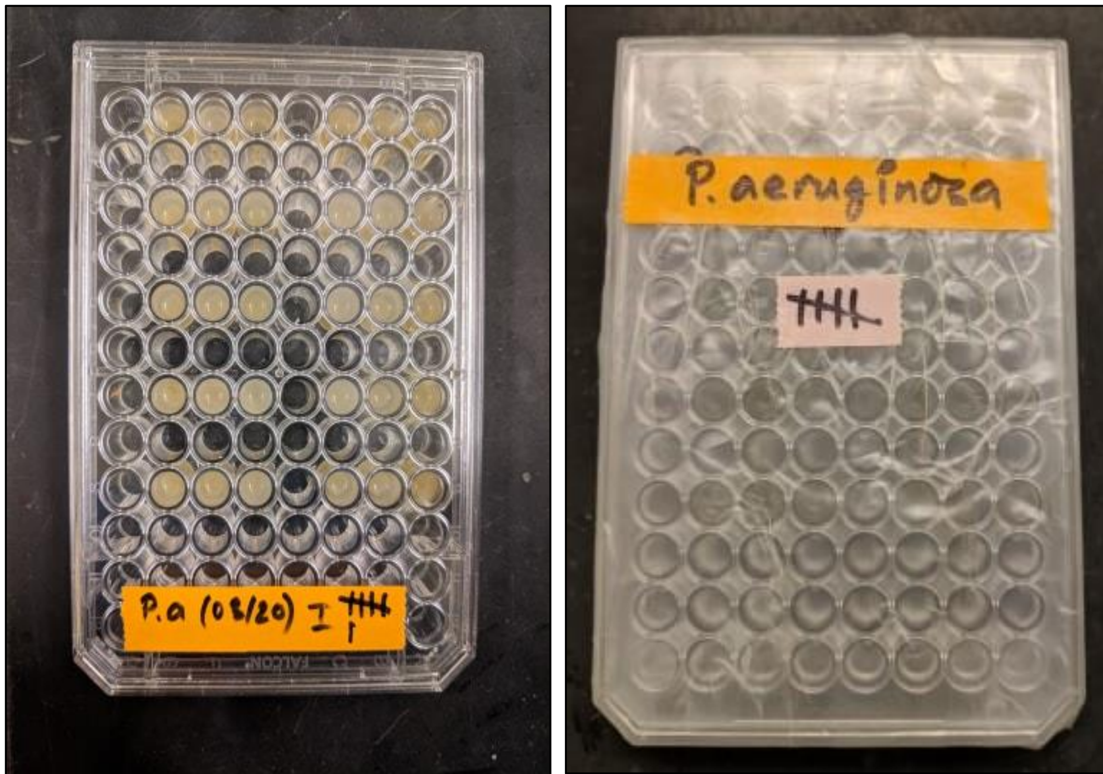


Figure 2. Growth of biofilms in microplates.

Washing biofilms and enzyme detachment of adhered cells from microplates

Magna plate washer was used to wash the microplates to remove loose planktonic cells from the biofilm. The plate washer was connected to different wash solutions depending on the need: 10% bleach solution, sterile de-ionized water, or 0.05 M tris buffer (pH 7.4), including several waste containers. Before washing the biofilm, the plate washer needles were sanitized by running the maintenance cycle using 10% bleach (2X times), sterile de-

ionized water (3X times) and tris buffer (2X times). Thereafter, the wash program was carried out in microplates containing biofilms.

The microplates were washed 3 times with tris buffer using the shake option to release loosely adhered cells. After 7 days of washing the biofilms and incubating with sterile media, the final wash was done with tris buffer using the plate washer. A trypsin enzyme solution was used for detachment of adhered cells from microplates to obtain a plate count enumeration from the biofilm. Trypsin solution (Cat: T4549; Sigma-Aldrich, St. Louis, MO, USA) was diluted with phosphate buffered saline (137mM NaCl, pH 7.4; sc-24946 Santa Cruz Biotechnology, Inc.) in the ratio 1:3 (~500 U/ml) and transferred into the experimental wells.

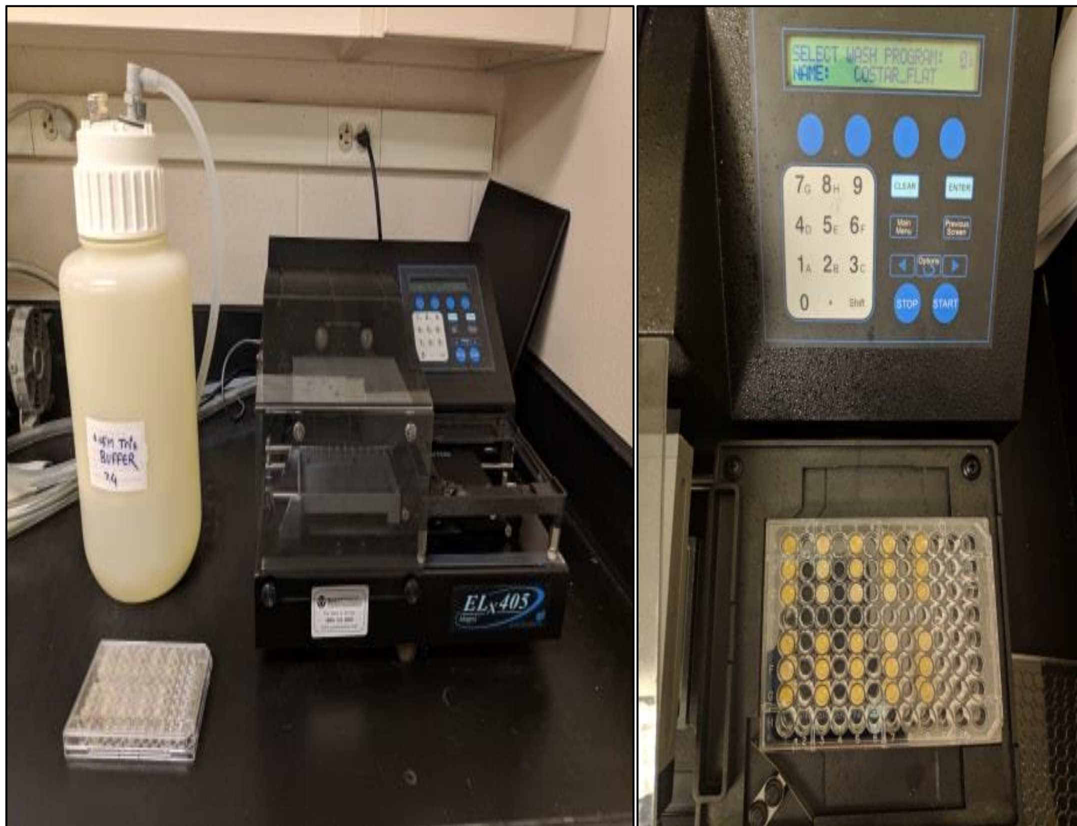


Figure 3. Washing of biofilms in Biotek Magna plate washer.

After the addition of trypsin, microplates were sealed with parafilm and incubated for 1 hour at 37°C. Finally, to get detached cell counts, the solutions from the wells were further diluted (200 µl of well suspension was transferred to 1800 µl of buffer solution followed by serial dilution) and plated on TSA plates and incubated at 30°C for 24 hours.

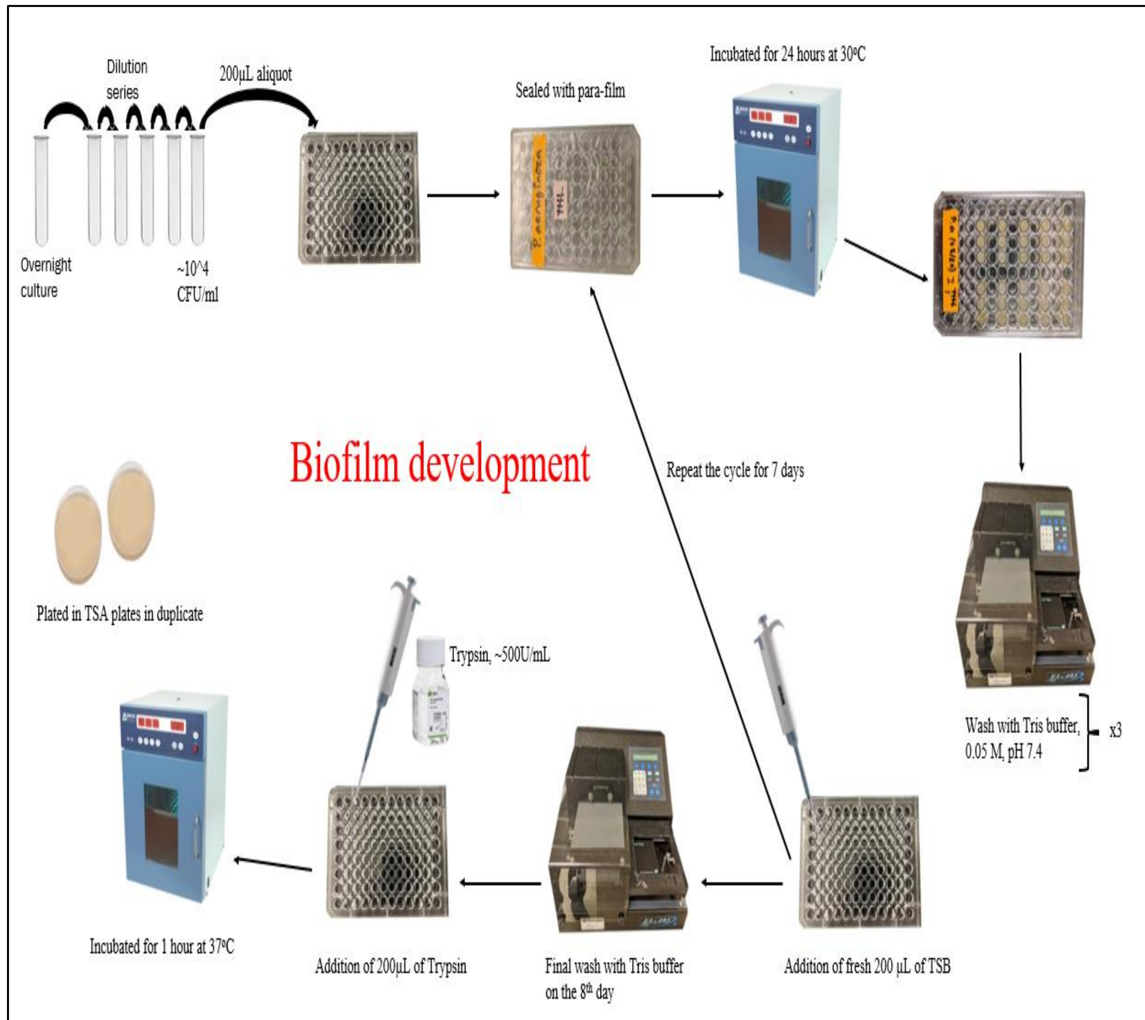


Figure 4. Development of 7-day extended robust biofilms.

Microplate biofilm sanitizer assay

Decon7 sanitizer solution (Decon™ Seven Systems, Scottsdale, AZ, USA) was used in our studies and it was composed of three parts: A surfactant (quaternary ammonium compound), an oxidizer (hydrogen peroxide) and an accelerator (diacetin). These three parts were mixed in the ratio 2:2:1 according to the manufacturer's instructions to form the stock solution. The concentrate was diluted to 5% and 10% concentrations of the stock solution to prepare 5% and 10% working solution for assessing its efficacy against biofilms.

Figure 5. Decon7 used for sanitizer assays.

Different microplates were used for each Decon7 application time of 1, 5, 10, and 20 minutes. After 7 days of growing biofilm, microplates were washed 3X times with sterile tris buffer (0.05M, pH 7.4). Treatments with Decon7 were then applied to the various

biofilms (5 different strains: 2 strains of *Pseudomonas* and 3 strains of *Staphylococcus*). Decon7 (200 uL, both 5% and 10%) was added into the appropriate wells and treated for 1, 5, 10, and 20 min accordingly. Treated microplates were washed with tris buffer (0.05M, pH 7.4) and 200 µL of Dey-Engley (D/E) neutralizing broth buffer (Hardy Diagnostics, Santa Maria, CA, USA) was added and left for 5 min to neutralize any potential residual Decon7. After treatment for 5 min with D/E neutralizing broth, microplates were washed with tris buffer (0.05 M. pH 7.4). Trypsin solution (250 uL; ~500 U/ml) was then added into the wells of microplates for enzymatic detachment of attached/residual viable bacterial cells. Microplates were sealed, incubated at 37ºC for one hour, plated on TSA plates in duplicate, and incubated at 30ºC for 48 hrs and enumerated. Microbial populations were converted to log denomination and plotted vs time (of treatment).

Figure 6. Sanitizer treatment (Decon7) and bacterial recovery through enzymatic detachment.

Biofilms in worker’s boots from slaughterhouse

A Sponge-stick (3M™) was used to swab the biofilm present on boots of workers in the FAPC meat processing plant. Trypsin solution (1 ml; 500 U/ml) was added to facilitate the detachment of bacterial cells in the biofilm. Six pairs of boots in three replicates were swabbed, turning swab stick over, and then breaking the sponge off into a sampling bag.

Figure 7. Sponge-stick used for swabbing biofilms from worker's boots.

The sampling bag was stomached, and plated on TSA plates. Bacteria isolated from worker’s boots were identified by 16S rRNA PCR and DNA sequencing. The effectiveness of Decon7 on the boot biofilm was examined using a liquid spray containing 10% concentration of Decon7 solution and sprayed (~14 sprays/boot~ 18 ml with a manual hand spray bottle) over those boots. After waiting 5 minutes after spraying, the boots were swabbed with a sponge stick containing trypsin solution. The sponge stick was stomached and then plated in TSA plates in duplicate.

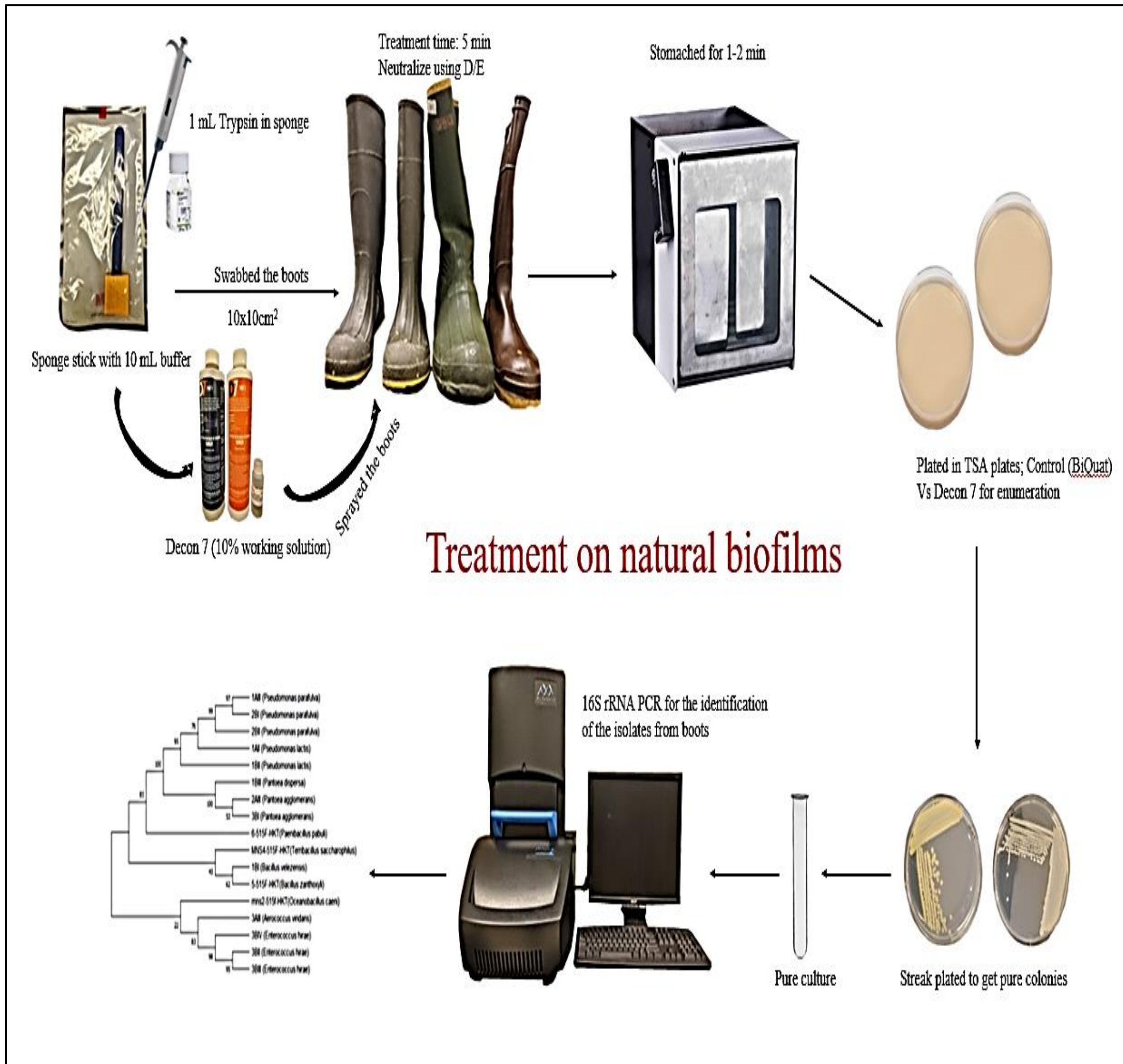


Figure 8. Treatment of Decon7 on natural mixed biofilms and identification of isolates from natural biofilms of boots.

16S rRNA PCR and DNA sequencing

The bacteria obtained from the workers' boots were streaked plated and isolated in a pure culture form. The cultures was grown in TSB at 30°C overnight and centrifuged (6000 × g, 5 °C) for 10 minutes. The supernatant liquid was discarded and freezing storage solution was transferred into the pellet. It was vortexed and 4 mL of each cell suspensions was transferred into vial and stored at -80°C. Frozen cultures were revived by thawing and transferring 100 µL to 9 ml TSB and incubated overnight at 30°C.

The overnight cultures were vortexed and 1 mL of those cultures were pelleted in 1.5 mL Eppendorf tube by centrifuging (12000 × g, 1 min). The supernatant was discarded and cells were suspended by adding 0.5 mL of tris buffer (50mM, pH 7.4) followed by centrifugation (repeated three times). After a third pelleting, the cell pellet was suspended with 200 µL of tris buffer (50mM, pH 7.4). Nitric acid washed glass beads were added to the conical bottom tube containing the cell pellet. It was vortexed for 2 min in an Eppendorf tube-holding adapter followed by 4 min on ice (repeated three times). This was further centrifuged at 12000 × g for 2 min to extract the DNA and 50-100 µL of the extracted DNA solution was recovered into a new sterile tube.

The extracted DNA was quantified using a Nano-drop 1000 spectrophotometer (ND-1000, Thermo scientific) by measuring the nucleic acid present (wavelength= 230nm). The extracted DNA was diluted with a nuclease-free water to a working concentration of ~1 ng/µL. For PCR amplification, the sample was prepared by adding 5 µL (~1ng/ µL) of DNA template, 12.5 µL of Promega master mix, primers (1 µL each 515-F and 1391-R, 0.5 µM) and 5.5 µL of nuclease free water. The samples were loaded into the plates in a thermocycler and programmed to run 16S RT-PCR. The parameters are programmed as

initial denaturation (95ºC/4 minutes), denaturation (94ºC/30seconds), annealing (50ºC/30 seconds) and, extension (72ºC/1 minute). The steps are repeated for 39 times and samples were held at 4ºC until purification.

Figure 9. Thermocycler for running 16S rRNA PCR.

The PCR product obtained was purified using Epoch GenCatch™ advanced PCR extraction kit and sent to the OSU DNA Core Facility for DNA sequencing. After sequencing, Mega-X software (Build# 10190812-x86_64, Penn State University) was used for sequence alignment and inferring phylogenetic trees of the isolated bacterial population obtained from the worker's boots. Maximum likelihood method was used for analysis to test the phylogeny using bootstrap method.

Comparison of antimicrobial activity of Decon7 and Bi-Quat against bacterial isolates from worker's boots

Bi-Quat, a first generation QAC, has been used in the FAPC slaughterhouse as a sanitizing agent for >20 years and we wanted to examine the antimicrobial activity differences between Bi-Quat and Decon7. A soft agar overlay technique was used to screen the effect of Bi-Quat and Decon7 sanitizers against the *Pseudomonas* and *Staphylococcus* strains that were used previously to form laboratory biofilms as well as against bacteria isolated from worker's boots: *Pseudomonas lactis, Pseudomonas parafulva, Pantoea dispersa, Pantoea agglomerans, Aerococcus viridans, and Enterococcus hirae*. Each bacterial strain (100 µL) was grown overnight in TSB at 30°C and 50 µL of overnight culture was added to soft agar of 5 mL (0.75%).

Figure 10. Comparison of Decon7 and Bi-Quat for antimicrobial activity.

The soft agar with inoculated culture was poured on top of prepoured TSA plates (1.5%) and allowed to sit to create a seeded soft agar overlay. Decon7 (10% i.e. 1280 ppm) and Bi-Quat (1000 ppm) of 200 µL were poured in separate wells in microplates. Then both of the sanitizers were serially diluted by transferring 100 µL of full strength solution (working

solution) from one well to 100 µL of sterile water into another well. Previously prepared soft agar seeded overlay were divided into 16 pie sections over two petri plates to spot test the sanitizer's activity. Antimicrobials (5 µL of sanitizer dilutions) were spotted over each section. The full strength working solution was spotted on section 1 followed by subsequent dilutions on sections 2, 3 and so on. The spotted samples were allowed to sit for 10 minutes and then incubated at 30ºC for 24 hours. The antimicrobial activity was then observed for each strain against the two sanitizers.

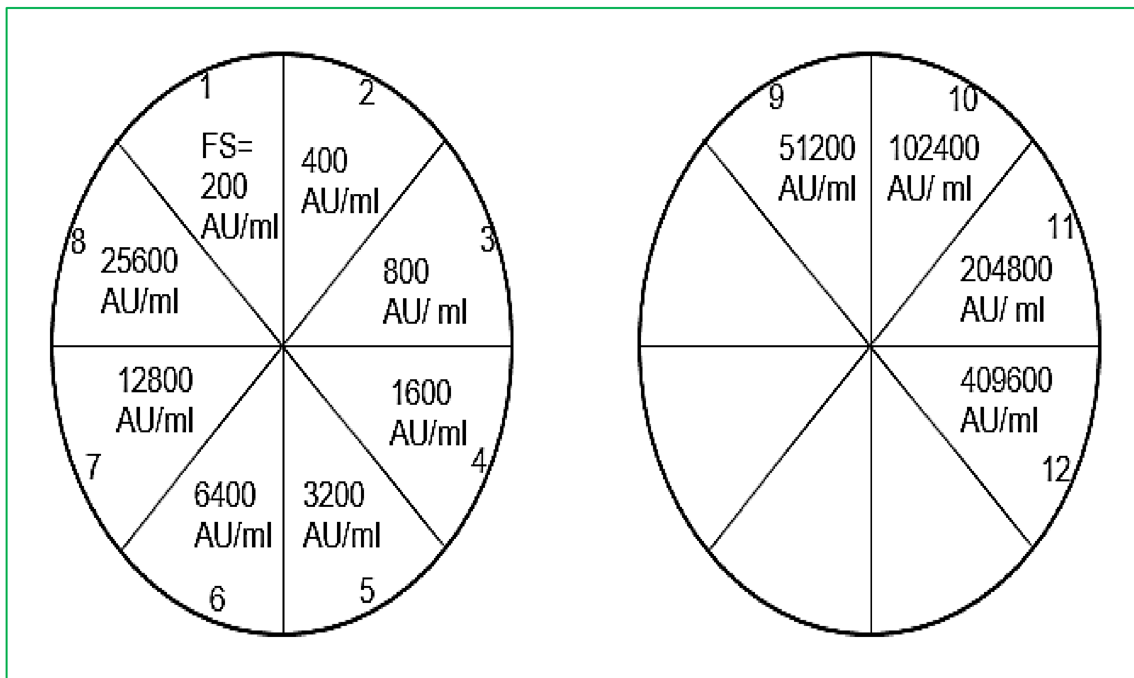


Figure 11. Antimicrobial activity enumeration: 5 µl for each spot, which is 1/200th of 1ml was spotted on each pie section. The last spot showing a faint zone of inhibition was regarded as the endpoint. For comparison, 'activity' was attributed as 'arbitrary units' of

Statistical Analysis

Each trial was performed in triplicate as a separate and independent experiment that made use of separately inoculated cultures and plating media. The effect of sanitizers on biofilms over different time (time-series plots) was statistically analyzed using repeated measures one-way analysis of variance (RM-ANOVA) with the help of Holm-Sidak test for multiple pairwise comparisons. The repeated measures involves the statistical comparison of one treatment curve to the another treatment curve. The difference in biofilm generation using treated and untreated plates was statistically analyzed using One Way ANOVA and Holm-Sidak method was used for pairwise multiple comparison procedures within each bacterial strain. The data displayed is the average of triplicate replications and error bars represents the standard deviation of the mean. Data treatments with different letters are significantly different ($p < 0.05$); treatments with the same letter are not significantly different ($p > 0.05$).

CHAPTER IV

RESULTS

Enzyme detachment of biofilms and treated vs untreated plates

Prior to using biofilms in a sanitizer assay, the biofilm generation using both cell culture treated plates and untreated plates were examined. The treated plates developed ~1- log higher biofilm growth compared to the untreated plates (Figure 12). The treated plates facilitate the attachment of biofilm bacteria to the wells and were therefore used in this study.

Figure 12. Comparison between 96-well cell cultures treated plates and untreated plates. The bar represents the means of triplicate replications and error bars represent the standard deviations from the means. Treatments with different letters are significantly different and treatment with same letter are not significantly different (pairwise multiple comparison procedures within each bacterial strains. Holm-Sidak method, Overall significance level = 0.05). Treatments with different letters are significantly different (One Way-ANOVA, $p < 0.05$); treatments with the same letters are not significantly different (One Way-ANOVA, $p > 0.05$).

Effect of Decon7 on biofilms of *Pseudomonas* and *Staphylococcus* strains

Decon7 is a new generation QAC sanitizer and consists of a 3-part solution formulation: a surfactant (benzyl-C12-C16 alkyl dimethyl chlorides), an oxidizer (hydrogen peroxide) and an accelerator (diacetin). Decon7 was used in a lethality assay on biofilms generated with five strains; *Pseudomonas aeruginosa 1, Pseudomonas aeruginosa 2, Staphylococcus aureus* PMM 174C1, *Staphylococcus aureus* PMM 169C8 and *Staphylococcus aureus* PMM 854HS-7 at both 5% and 10% concentrations.

Decon7 showed ~4-5 log reduction within the first 1 minute of treatment at both 5% and 10% on most of the strains and a greater reduction was observed for 10% Decon7 than for 5% Decon7 (Figures 13,14,15,16 & 17).

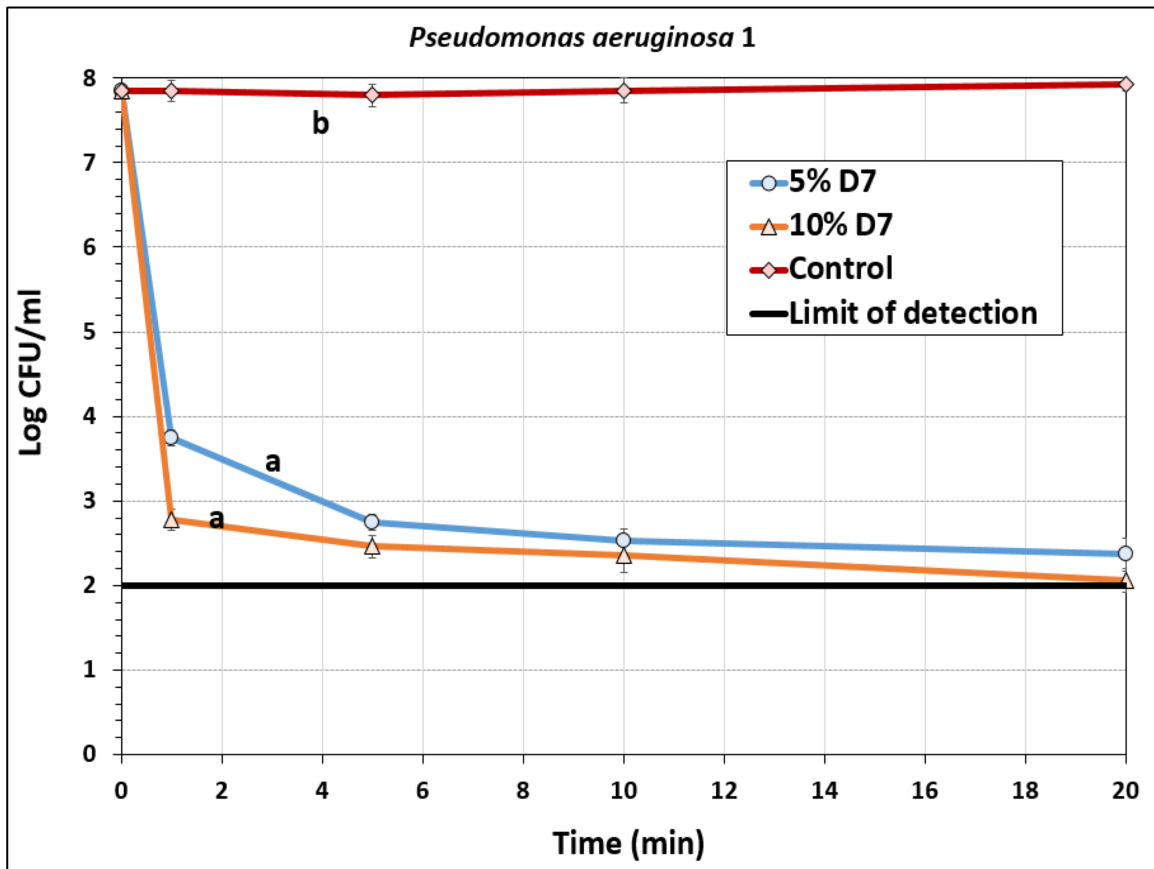


Figure 13. Biofilm microplate lethality assay on *Pseudomonas aeruginosa* 1, 7-day biofilms challenged with 5% and 10% solutions of Decon7 sanitizer for up to 20 min. Data points represent the means of triplicate replications and error bars represent the standard deviations from the means (some error bars may be hidden by the large symbols). Treatments with different letters are significantly different (RM-ANOVA, $p < 0.05$); treatments with the same letters are not significantly different (RM-ANOVA, $p > 0.05$). The bold black line represents the microbial limit of detection.

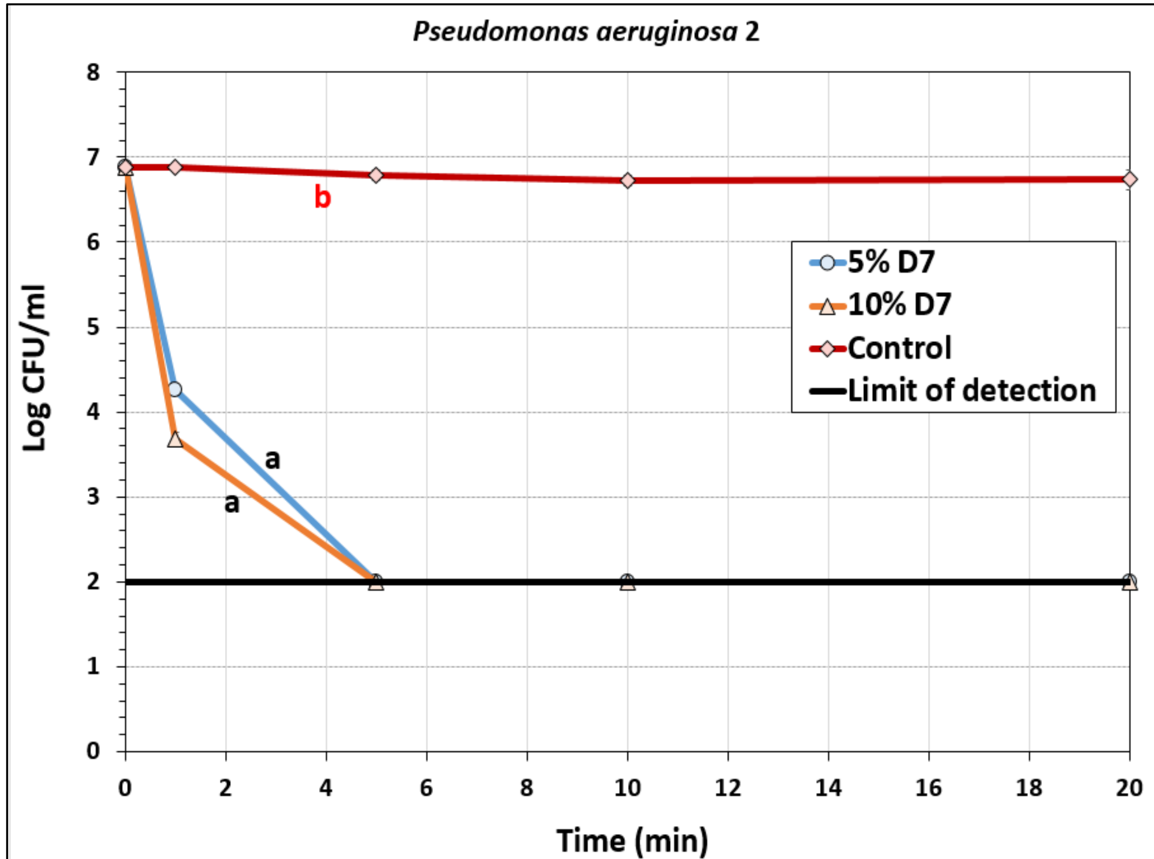


Figure 14. Biofilm microplate lethality assay on *Pseudomonas aeruginosa* 2, 7-day biofilms challenged with 5% and 10% solutions of Decon7 sanitizer for up to 20 min. Data points represent the means of triplicate replications and error bars represent the standard deviations from the means (some error bars may be hidden by the large symbols). Treatments with different letters are significantly different (RM-ANOVA, $p < 0.05$); treatments with the same letters are not significantly different (RM-ANOVA, $p > 0.05$). The bold black line represents the microbial limit of detection.

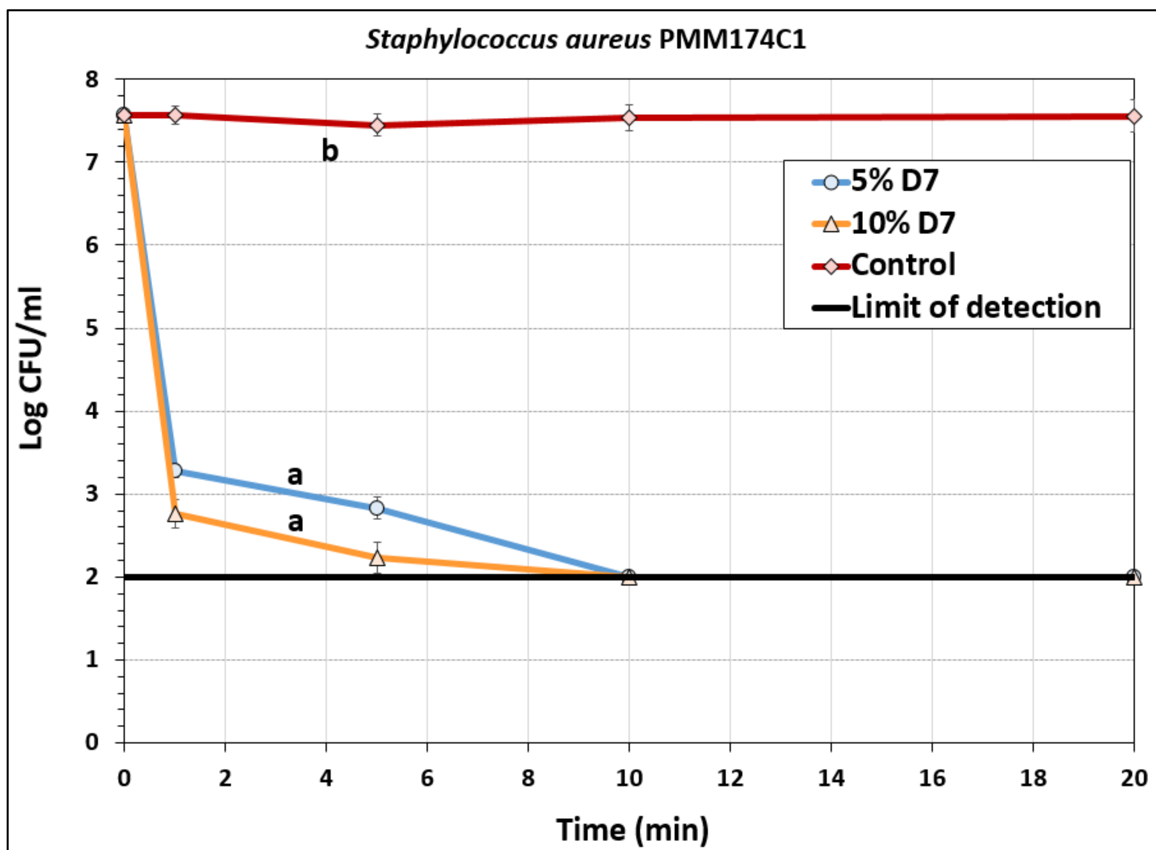


Figure 15. Biofilm microplate lethality assay on *Staphylococcus aureus* PMM 174C1, 7-day biofilms challenged with 5% and 10% solutions of Decon7 sanitizer for up to 20 min. Data points represent the means of triplicate replications and error bars represent the standard deviations from the means (some error bars may be hidden by the large symbols). Treatments with different letters are significantly different (RM-ANOVA, $p < 0.05$); treatments with the same letters are not significantly different (RM-ANOVA, $p > 0.05$). The bold black line represents the microbial limit of detection.

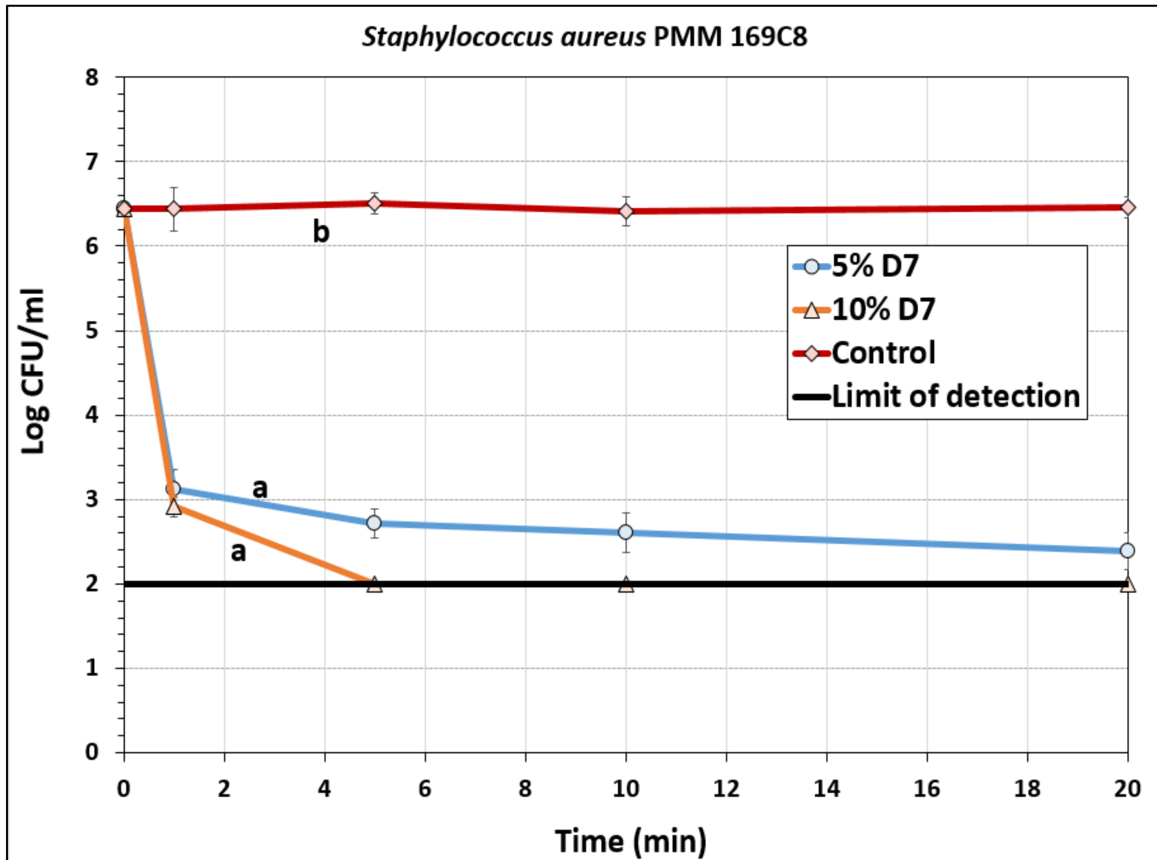


Figure 16. Biofilm microplate lethality assay on Staphylococcus aureus PMM 169C8, 7-day biofilms challenged with 5% and 10% solutions of Decon7 sanitizer for up to 20 min. Data points represent the means of triplicate replications and error bars represent the standard deviations from the means (some error bars may be hidden by the large symbols). Treatments with different letters are significantly different (RM-ANOVA, $p < 0.05$); treatments with the same letters are not significantly different (RM-ANOVA, $p > 0.05$). The bold black line represents the microbial limit of detection.

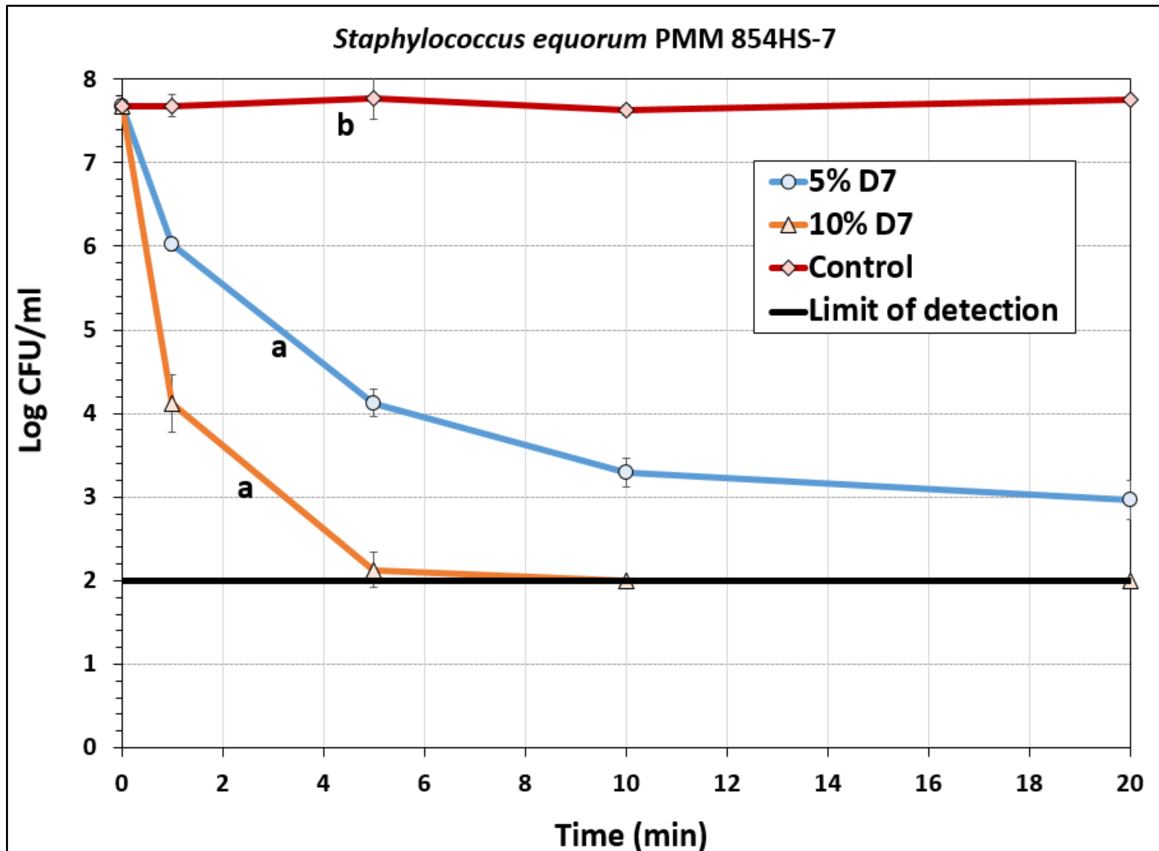


Figure 17. Biofilm microplate lethality assay on *Staphylococcus equorum* PMM 854HS7, 7-day biofilms challenged with 5% and 10% solutions of Decon7 sanitizer for up to 20 min. Data points represent the means of triplicate replications and error bars represent the standard deviations from the means (some error bars may be hidden by the large symbols). Treatments with different letters are significantly different (RM-ANOVA, $p < 0.05$); treatments with the same letters are not significantly different (RM-ANOVA, $p > 0.05$). The bold black line represents the microbial limit of detection.

Treating biofilms on worker's boots from the FAPC slaughterhouse with Decon7

The worker's boots from the FAPC slaughterhouse showed a high level of bacterial biofilms (~6 log CFU/inch^2) when swabbed with trypsin solution using a sponge stick and enumerated on TSA plates. We could achieve ~ 3-log reduction when boots were sprayed with Decon7 solution when combined with trypsin for detachment and enumeration (Figure 18). Six pairs of boots with three replicates each were assessed for evaluating the effectiveness of Decon7 sanitizer against natural biofilms found on worker's boots. These generic biofilms develop on a regular basis when workers move into the slaughterhouse and at each time nutrient media and water is added that enhances its formation. Bi-Quat sanitizer that has been used in the slaughterhouse for >20 years is not highly effective as it only selects for alkaliphilic organisms.

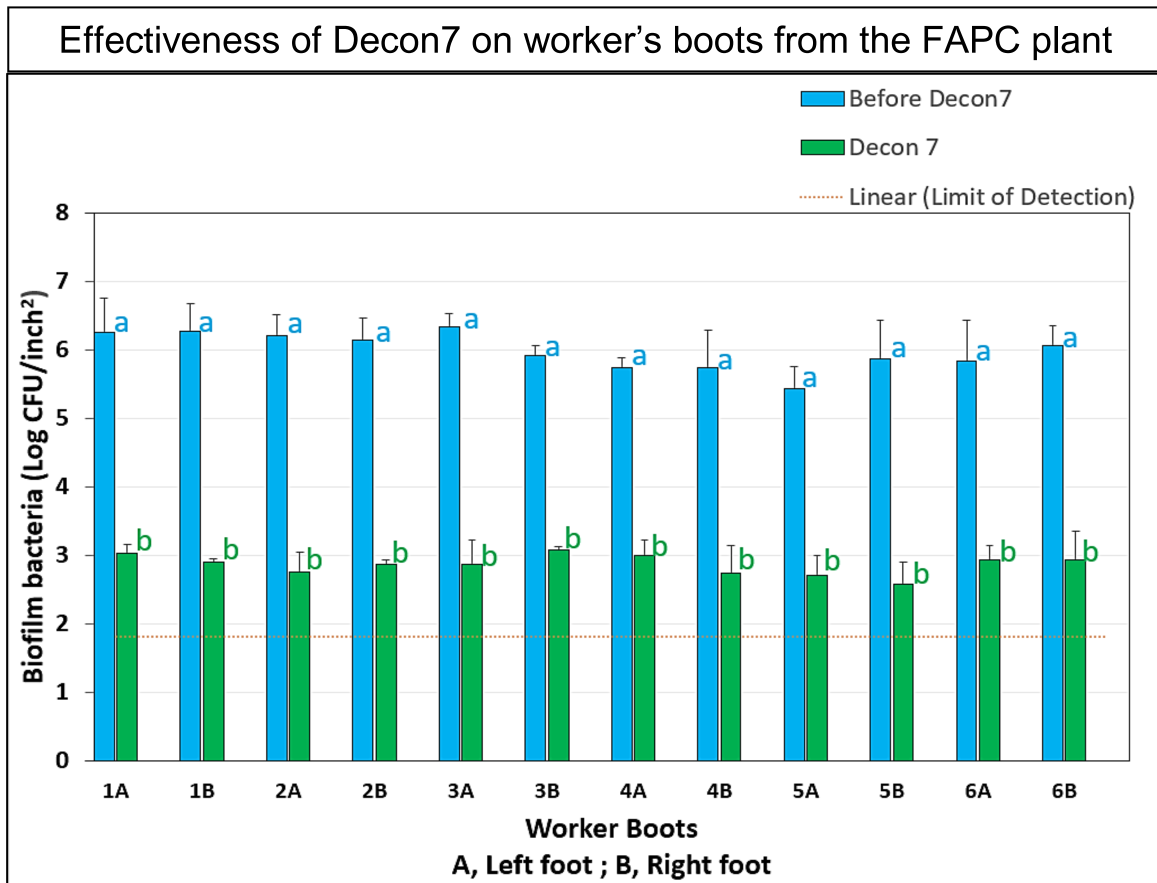


Figure 18. Enumeration of bacterial populations from encrusted biofilms on worker's boots. 'A' represents left foot and 'B' represents the right foot boot. Six pairs of boots (3 replicates) were assessed to test the sanitizer lethality and isolate bacterial strains. Data points represent the means of replication and error bars represent the standard deviations from the means. One Way ANOVA shows the differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($P = <0.001$). Treatments with different letters are significantly different and treatment with same letter are not significantly different (pairwise multiple comparison procedures: Holm-Sidak method, overall significance level = 0.05).

DNA sequencing and sequence alignment

We used 16S rRNA PCR to identify the organisms present on the biofilms of worker's boots. Seven organisms were isolated and characterized namely: *Pseudomonas parafulva, Pseudomonas lactis, Pantoea dispersa, Pantoea agglomerans, Aerococcus viridans, Bacillus velezensis, Enterococcus hirae, Bacillus zanthoxyli, Oceanobacillus caeni* and *Teribacillus sacchrophilus.* These organisms are characterized based on the percentage identity obtained when the sequences obtained from DNA sequencing were subjected to BLAST search and matched with the DNA sequence in the database. Percent identity is used to describe the ratio of identical nucleotide bases shared by the query and reference sequences (from the database) to the number of nucleotide bases that were sequenced. It is acceptable to classify an organism to genus and species if it has a percent identity score of ≥97% and ≥99% (Reller, Weinstein, & Petti, 2007). Figure 19 represents the phylogenetic tree of bacterial isolates obtained from worker's boots. The phylogenetic tree was inferred by using maximum likelihood as a statistical method and bootstrap method was used as the test of phylogeny with a bootstrap number of replications being 1000. The substitution type was nucleotide and Jukes Cantor model was selected as the substitution model.

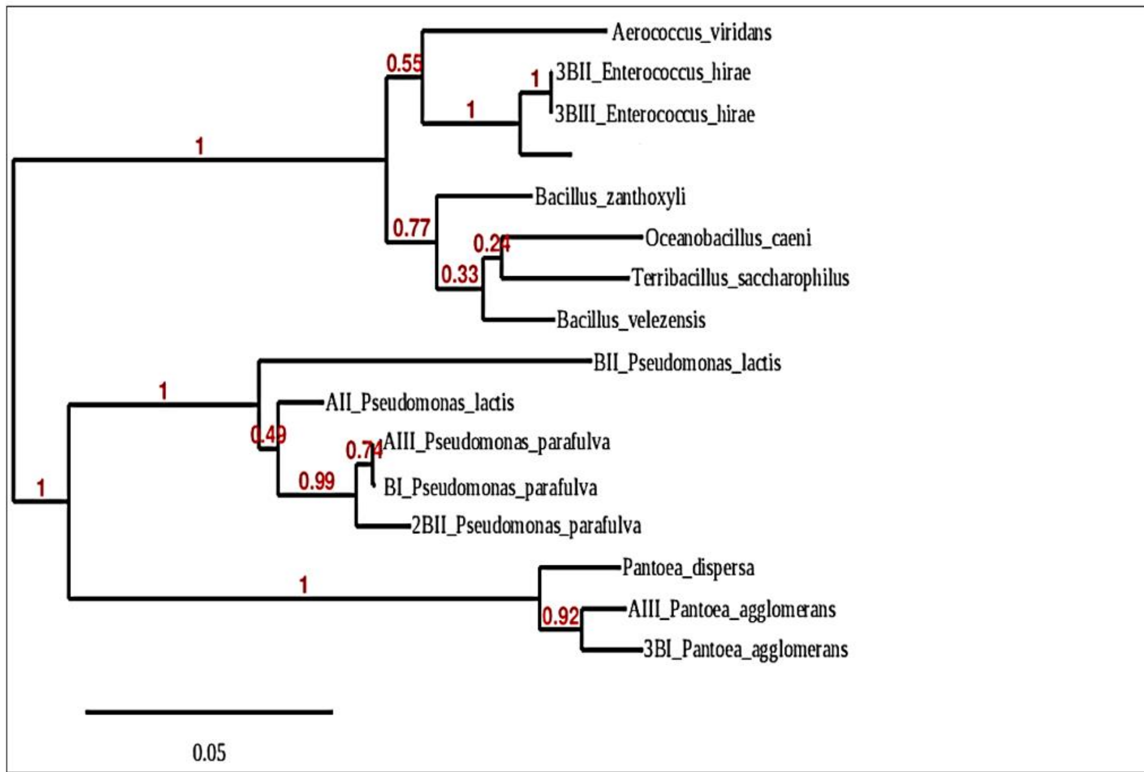


Figure 19. Phylogenetic tree of isolated bacterial strains from the natural biofilms found on worker's boots, inferred by using maximum likelihood method using bootstrap consensus tree. The value at the nodes represent bootstrap value (i.e. percentages based on 1000 replicates that signifies how many times out of 100, the same branch was observed when repeating the phylogenetic reconstruction on a re-sampled set of the data). The length of the branches (horizontal line) represent the genetic distance between the organisms. The value 0.05 refers to the nucleotides/site in the alignment that gives the measure of the scale of genetic distance between each bacteria.

Antimicrobial activity of Decon7 vs Bi-Quat against the bacterial isolates derived from the biofilms of worker’s boots

The antimicrobial activity of both Decon7 and Bi-Quat were assessed by using a soft agar overlay assay using various biofilm bacteria as test organism. It showed that the activity of Decon7 is greater than that of Bi-Quat. Additionally, the strains isolated from worker’s boots showed less sensitivity to the sanitizers as compared to the strains that were previously used as a lethality assay of Decon7.

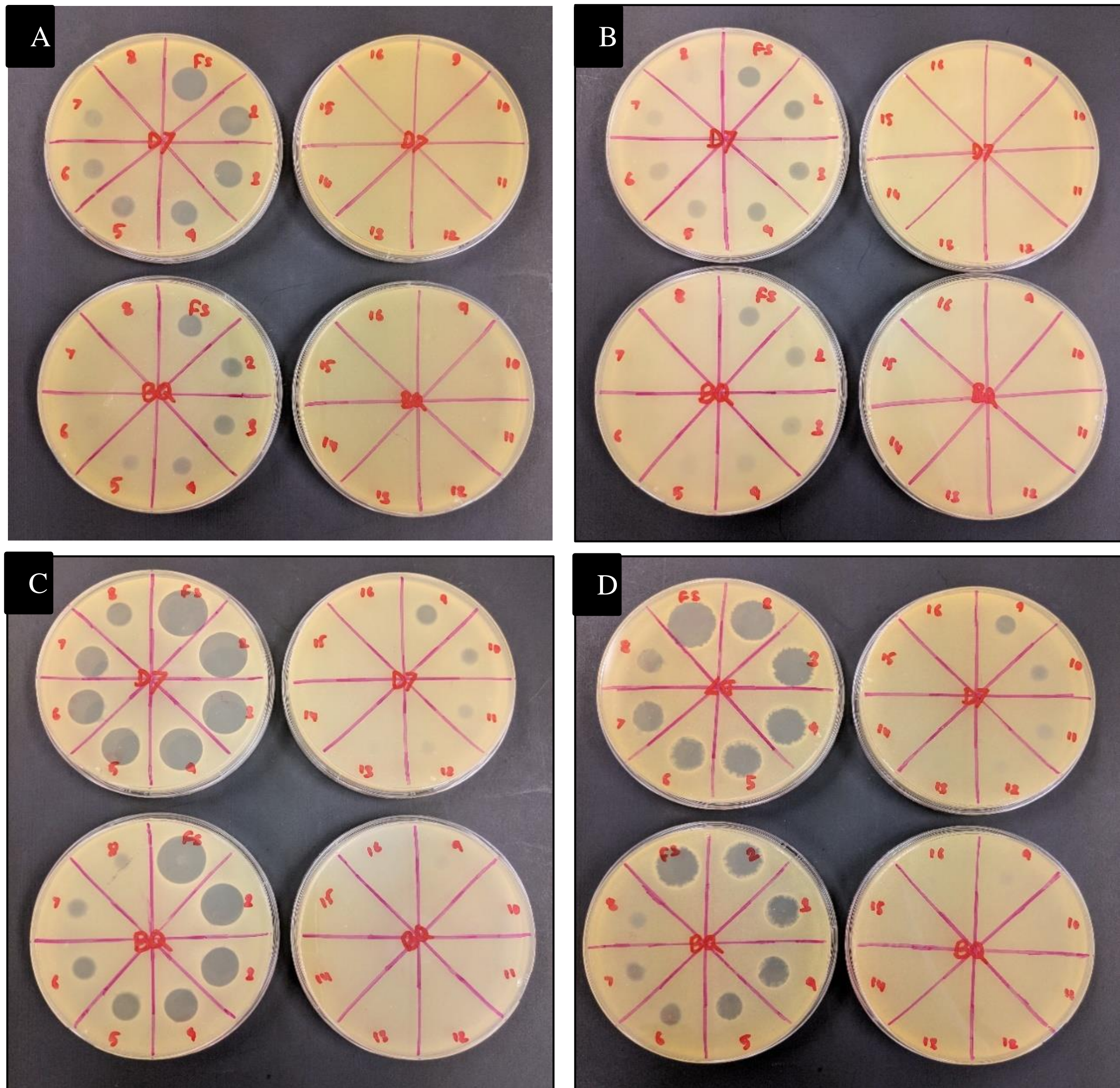
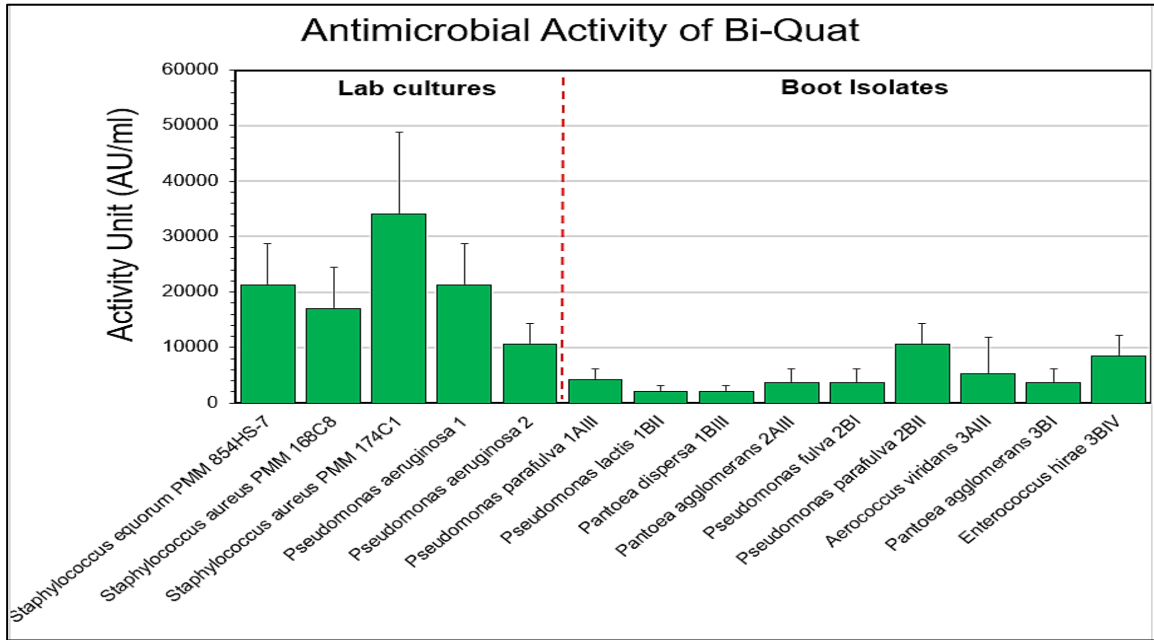


Figure 20. Antimicrobial activity of Bi-Quat (bottom) and Decon7 (top) against the bacterial strains used in lethality assay and bacterial isolates from the worker's boots. A: *Pantoea agglomerans*; B: *Pseudomonas lactis*; C: *Staphylococcus equorum* PMM 854HS-7*; D: Pseudomonas aeruginosa*



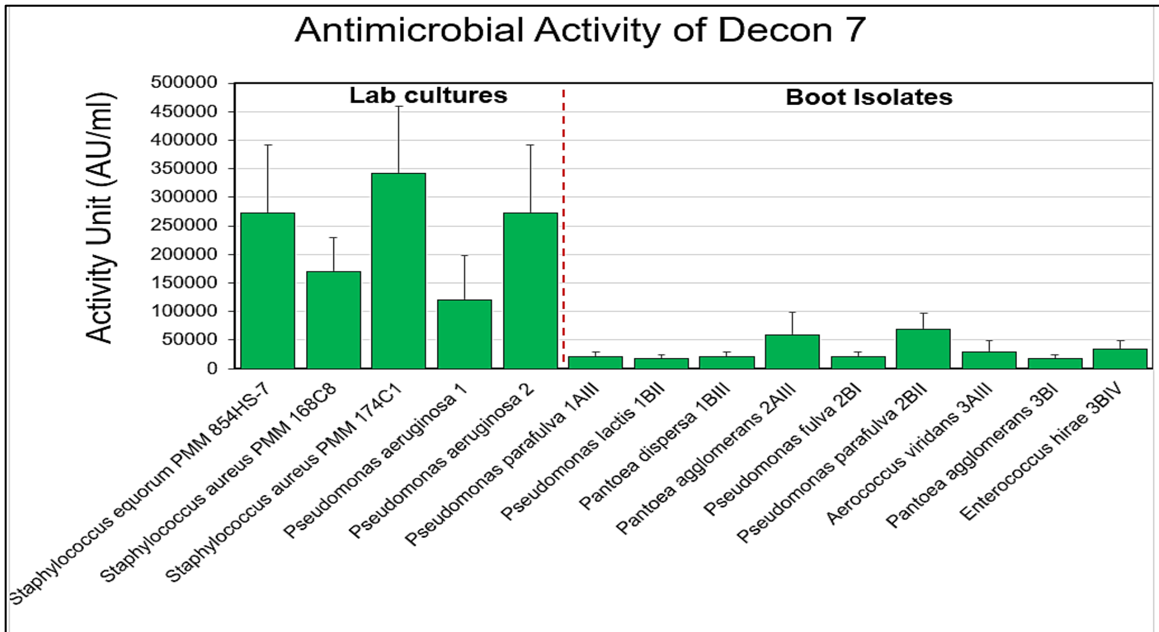


Figure 21. Comparison of antimicrobial activity of serial dilutions of Bi-Quat or Decon7 sanitizer spotted on lawns of laboratory biofilm bacteria (left side) vs bacterial isolates from biofilm on worker's boots (right side) to the sanitizer treatment.

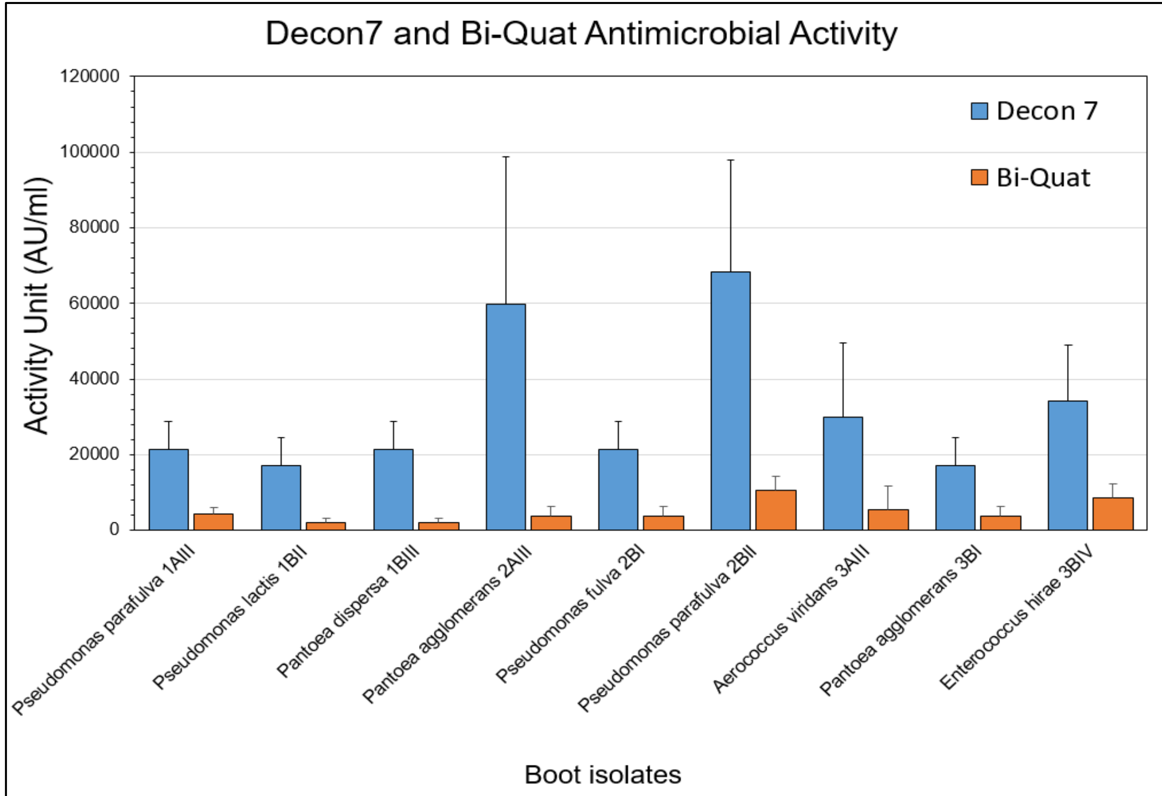


Figure 22. Comparison of antimicrobial activity of Decon7 and Bi-Quat (or sensitivity of the organisms to the sanitizers) against the biofilm bacteria found on worker's boots from the FAPC meat processing facility

CHAPTER V

DISCUSSION

This work was carried out as an extension of prior research from our lab on biofilms and sanitizers. We used flat-bottom microplates (96-well) and daily repeated washing with a plate washer that has a plate-shaking action to remove planktonic/loose cells. This was followed by addition of fresh media to create a 7-day enhanced biofilm of *Pseudomonas aeruginosa* 1, *Pseudomonas aeruginosa* 2, *Staphylococcus aureus* PMM 171C1, *Staphylococcus aureus* PMM 169C8, and *Staphylococcus equorum* PMM 854HS-7. EPA requires these organisms to be used if sanitizer is going to state that it is effective against biofilms (Sanders, 2003). The use of treated cell culture plates gave a better ~ 1-log increase in biofilm development than when using untreated plates. The treated plates facilitates the adherence of biofilm bacteria to the bottom and sides of the 96-wells.

In prior studies, the use of untreated plates allowed the evaluation of the inherent ability of individual strains to adhere to allow the selection of strongly adherent strains. In this study, we are seeking to achieve the highest level of biofilm attachment to evaluate the Decon7 sanitizer. Biofilms develop in a stepwise process, beginning with attachment of individual cells (reversible) followed by secretion of polymeric substances that helps in binding of cells more firmly in a heterogeneous way. After binding together, a cluster of extracellular

polysaccharides (EPS), proteins, nucleic acids, fats, and water helps to develop a mature biofilm. This biofilm is irreversible and difficult to remove in commercial facilities. It further disperses its cells to initiate the formation of a new biofilm in new locations. One of the major factors in biofilm formation with *Staphylococcus aureus* is the expression of the icaA gene, which helps in the formation of a transcriptionally expressed product N-acetyl-glucosaminyl transferase (Abdallah et al., 2015). This enzyme is involved in the biosynthesis of an extracellular polysaccharide matrix that acts as a protective layer to protect the bacterial population from unfavorable environmental conditions (i.e., sanitizers). Diffusible organic signal molecules regulate gene expression by a process called "quorum sensing" to communicate among the bacterial cells in a biofilm. It helps in the regulation of water, nutrients and removal of waste products from the cells. This cooperation among the cells increases the level of protection and resistance to the antagonistic environment (Donlan, 2002). In our research, developing a robust biofilm of each bacterial strain was mediated by continuous washing with tris buffer (to help remove the planktonic and dead bacterial cells) and adding fresh nutrient medium (TSA broth) each day for 7 days. The bacterial population in our biofilm achieved a ~6.5-7.5-log CFU/ml level with treated plates as determined from cell enumeration after enzymatic detachment. After treating the 7-day old biofilm with Decon7, we observed approximately ~4-5 log reduction in all the strains tested within the first 1 minute of treatment using 10% concentration. The Decon7 that we use is a second-generation QAC sanitizer; it includes hydrogen peroxide and quaternary ammonium chloride (benzyl-C12-C16 alkyl dimethyl chlorides) along with diacetin as a booster. Decon7 has 3.2% QAC in the concentrate as a surfactant. A cationic surfactant like QAC has been found to have a cleaning (cell removal)

activity (McEldowney & Fletcher, 1987). In actual practice, Decon7 is applied as a foam that provides greater interaction time on the surfaces in any food processing facilities like an abattoir. QAC's are found to be effective against both Gram-positive and Gram-negative bacteria (Carsberg, 1996). The surfactant acts on the phospholipid components in the cytoplasmic membrane that causes distortion and lysis causing osmotic stress. Another compound in Decon7 is hydrogen peroxide (H_2O_2) and acts as an oxidizer, which is 7.9% in the concentrate. It is a broad-spectrum sanitizer and is innocuous to the environment. Hydrogen peroxide releases hydroxyl free radicals (•OH) as an oxidant. These radicals are unstable molecules that act upon lipids, proteins and DNA specially targeting the double bonds and sulfhydryl groups within the cell wall component and damages the cell (Seymour S Block, 2001).

Bi-Quat (alkyl dimethyl benzyl ammonium chloride) is an early generation QAC that has been used for a long time as a regular sanitizer in the FAPC Meat Processing Pilot Plant for >20 years. We found high levels of biofilm bacteria on all boots tested from student worker's in the FAPC slaughterhouse facility. Decon7 was used on the boots of student worker's and we could achieve ~ 3-log reduction. The regular use of Bi-Quat with its lower level of antimicrobial activity might have allowed the development of biofilms that were resistant to further Bi-Quat treatment. The microorganisms isolated from boots biofilms were regularly subjected to the use of Bi-Quat, which is an alkaline-based sanitizer. Thus, these bacteria could experience habituation to alkaline conditions, and this goes together with the increase in resistance that was observed. A number of studies have indicated that some of our isolated bacterium to be alkaline resistant. *Enterococcus hirae* previously known as *Streptococcus facialis* (Gaechter, Wunderlin, Schmidheini, & Solioz, 2012) are

described as alkaline resistant (Downie & Cruickshank, 1928; Graham & Lund, 1983; Krulwich & Guffanti, 1989). Similarly, *Bacillus* is also reported to be highly resistant to alkaline solutions (Krulwich & Guffanti, 1989; Nielsen, Fritze, & Priest, 1995). Although there is less information on alkaliphilic *Pseudomonas* species, they are found to utilize the cytochrome *c* that has an excellent electron retaining ability. In addition, the periplasmic space of *Pseudomonas* contains H^+ and electron condenser that accumulates H^+ which protects the bacterium from the extracellular alkaline environment (Matsuno & Yumoto, 2015). There are many studies related to developing resistance against antibiotics in microbial organisms when sanitizers are used on a regular basis. Gram-positive microorganisms like *Staphylococci* have been reported to develop resistance against QAC in food processing systems (Heir, Sundheim, & Holck, 1995). Likewise, when subjected to biocidal use, both Gram-positive and Gram-negative bacteria were capable of enhancing antimicrobial resistance (Kampf, 2018, 2019). Similarly, oxidizing agents such as hydrogen peroxide when used at sub-lethal concentrations develop resistance to ciprofloxacin in *Staphylococcus aureus* (Wesgate, Grasha, & Maillard, 2016). After disinfectants are left on the surface of food processing industries before rinsing with water there is a possibility that the microorganism remains into a prolonged exposure to that disinfectant which may lead to a slow development of its resistance along with co-selection of antibiotic resistant genes (Sidhu, Sørum, & Holck, 2002). In addition, there is a possibility that the antimicrobial resistant microorganisms could transfer their resistance genes to the receptive foodborne pathogens. 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.

Conclusion

Compared to other sanitizers that we have used against *Listeria monocytogenes*, *E. coli* O157:H7, and *Salmonella* Montevideo, Decon7, a next-generation sanitizer, provides the greatest reduction in the shortest time of application (Aryal et al., 2019). We have further observed effective reduction of 2 strains of *Pseudomonas aeruginosa* that were problem isolates from commercial liquid egg pasteurization facilities as well as 2 strains of *Staphylococcus aureus*, and 1 strain of *Staphylococcus equorum*. Decon7 also showed a reduction of bacterial population when sprayed directly on worker's boots as compared to a first generation sanitizer like Bi-Quat. After doing a comparison of sensitivity on *Pseudomonas* and *Staphylococcus* strains used in the microplate lethality assay vs bacterial isolates recovered from the boot biofilms, we found that the boot isolates were more Bi-Quat resistant. Although sensitivity to Decon7 was also reduced in these strains, Decon7 was able to provide >3 log reduction of these bacteria in boot biofilms that could not be obtained with Bi-Quat. We can conclude that the repeated use of Bi-Quat imposed a strong selective pressure for alkaliphilic bacteria thus increasing sanitizer resistance in abattoir biofilms as determined by analysis of the bacteria from worker's boots. Based on our data, Decon7 has proven itself to be very effective against biofilms. It is also prudent for us to advocate alternating different sanitizer chemistries to decrease the potential occurrence of antimicrobial resistivity of bacteria to sanitizers that could develop into sanitizer resistant biofilms.

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VITA

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