Validation of Sanitizer Effectiveness Against *Staphylococcus* and *Pseudomonas* Biofilms, Natural Biofilms from Worker's Boots, and Correlation of Biofilm Bacteria to Sanitizer Chemistry

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ABSTRACT

Introduction: 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.

Purpose: The objectives of our study were to 1) confirm sanitizer effectiveness on biofilms of *Staphylococcus* and *Pseudomonas*, 2) validate sanitizer effectiveness on real-life samples of workers' boots from a slaughter floor environment, 3) identify biofilm bacteria from old boots in relation to sanitizer chemistry, and 4) evaluate enzymatic treatment to breakdown biofilms prior to sanitizer application.

Methods: A sanitizer that demonstrated superior effectiveness against E. coli O157:H7, Salmonella spp., and Listeria monocytogenes was applied at 2 concentrations against enhanced biofilms of 5 strains of *Staphylococcus* spp. and *Pseudomonas* spp. (as required by EPA) in 96-well 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.

Results: 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.

Significance: The data show that 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 occur.

Keywords: Biofilm, sanitizers, Decon7, *Pseudomonas*, *Staphylococcus*, food contact surfaces.

INTRODUCTION

Microorganisms can establish a persistent presence in food processing facilities by adhering to surfaces and then creating a biofilm that may contain many different microorganisms. 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 99.999% for food contact sanitization. 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.*

INTRODUCTION (Cont.)

Our laboratory research simulated the natural environment to create biofilms by repeatedly washing and re-supplying nutrient medium to microplate wells for an extended time. This method helps 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 allow for detachment to recover viable cells from those extended biofilms.

Prior studies compared 5 different sanitizers: Bi-Quat (a simple QAC sanitizer), 10-Chlor (hypochlorite based sanitizer), KC-610 (peroxyacetic acid based sanitizer), and Sterilex and Decon7 solutions (new generation QACs) against *L. monocytogenes, E. coli* O157:H7, and *Salmonella* Montevideo (Aryal and Muriana, 2020), and showed that Decon7 was the most effective sanitizer.

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 *Pseudomonas* and *Staphylococcus* to facilitate claims that Decon7 works against biofilms. Additional studies examined the practical application of Decon7 in 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 sanitizer chemistry by 16S rRNA PCR and DNA sequencing and a comparison test was carried out between Decon7 and Bi-Quat to see the antimicrobial activity against the strains isolated from processing plant.

MATERIALS and METHODS

Bacterial cultures and growth condition. Cultures were from the Muriana Bacterial Culture Collection:

1. *Pseudomonas aeruginosa* 1, *Pseudomonas aeruginosa* 2 that were problem isolates from egg pasteurization facilities.

2. *Staphylococcus aureus* PMM 174C1, *Staphylococcus aureus* PMM 169C8 and *Staphylococcus equorum* PMM 854HS-7.

The cultures were grown in Tryptic Soy Broth (TSB, BD, NJ, USA) at 30°C overnight and centrifuged (6000 × g, 5°C) for 10 min. The supernatant liquid was discarded and freezing medium (TSB + 10% glycerol) was used to resuspend the pellet and stored in glass vials -80ºC. Frozen cultures were revived by thawing and transferring 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 an independent experiment. Microbial enumeration was performed on Tryptic Soy Agar (TSA), plated in duplicate.

Growth of Enhanced Biofilms in Microplates. The overnight grown cultures of *Staphylococcus* and *Pseudomonas* were diluted to ~4 log CFU/mL in TSB. Falcon 96-well, clear, non-treated, flat-bottom microplates (Cat# 351172, Corning, NY, USA) were used to develop biofilms. A 200 µL aliquot of the culture was allocated into microplate. The microplate was then sealed with para-film to avoid evaporation and then incubated at 30°C for 24 hours. After 24 hours, the microplate was washed 3x 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 TSB was added to the wells containing attached bacterial cells. It was then sealed with para-film and incubated for 24 hours at 30°C.This cycle of growing, washing, and media renewal was repeated daily for 7 days to develop a robust biofilm of attached cells in the wells.

Washing Biofilms and Enzyme Detachment of Adhered Cells from Microplates. The Biotek Magna plate washer was used to wash the microplates to remove any form of planktonic cells from the biofilm. It was able to draw different wash solutions depending on need: 10% bleach solution, sterile de-ionized water, or 0.05 M Tris buffer pH 7.4.

MATERIALS and METHODS (Cont.)

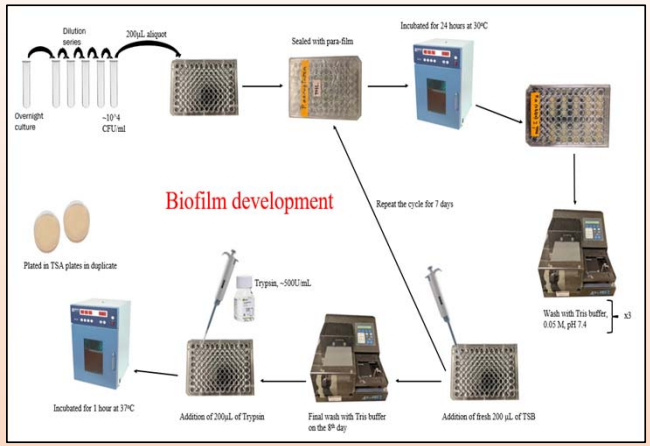


Figure 1. Development of 7-day extended biofilms.

Microplate Biofilm Sanitizer Assay. The Decon7 sanitizer (Decon™ Seven Systems, Scottsdale, AZ, USA) came in three parts: A surfactant (quaternary ammonium compound), an oxidizer (hydrogen peroxide) and an accelerator (diacetin).

Figure 2. Sanitizer treatment (Decon7) and bacterial recovery.

MATERIALS and METHODS (Cont.)

Biofilm bacteria from boots. Sponge-sticks with 1 mL Trypsin solution was used to swab the biofilm present in boots of workers from our in-house meat processing plant. The sponge swab was stomached and plated on TSA. Bacteria isolated from workers' boots were identified by 16S rRNA PCR and DNA sequencing.

Sanitizer Treatment of boots. A liquid spray of 10% Decon7 was prepared and sprayed over those boots. After 5 minutes of application, the boots were rinsed with buffer and swabbed with sponge stick containing Trypsin solution, stomached and then plated in TSA plates in duplicate.

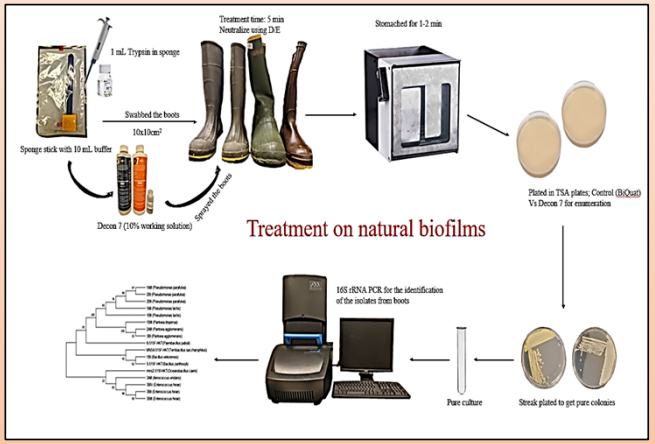
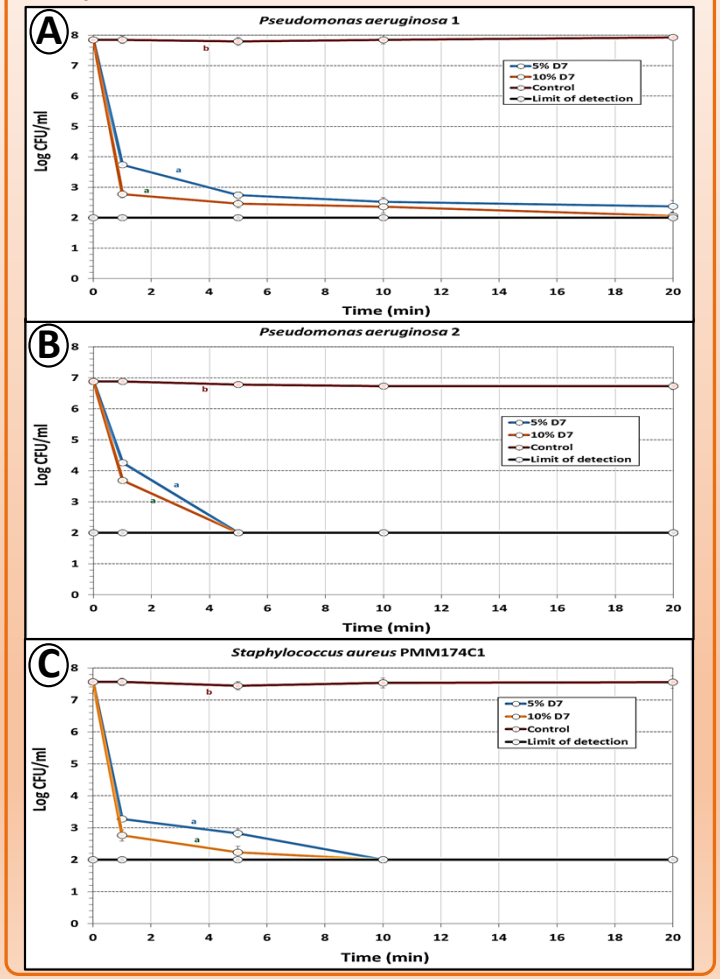


Figure 3. Treatment of Decon 7 on natural mixed biofilms

Comparison of Antimicrobial Activity of Decon7 and Bi-Quat against Bacterial Isolates from Workers' Boots. A soft agar overlay technique was used to screen the effect of Biquat and Decon7 sanitizers. The culture were grown overnight in TSB at 30ºC and 50 µL culture was added to 5 mL of soft agar (0.75%). The soft agar with inoculated culture was poured as a thin layer on TSA plates (1.5%) and allowed to sit until hard and marked into 16 'pie-sections' on two petri plates to test sanitizer activity. A 5-µL aliquot of each of the sanitizer serial dilutions was added into each pie section. Decon7 (10%; 1280 ppm) and Bi-Quat (1000 ppm) were serially diluted with sterile water.

RESULTS

Effect of Decon7 on Biofilms of *Pseudomonas* and *Staphylococcus* strains Decon7 was used in a lethality assay on biofilms of 5 test strains at both 5% and 10% concentrations. Decon7 showed ~5 log reduction within first 1 minute of treatment at both 5% and 10% on most of the strains.



RESULTS (Cont.)

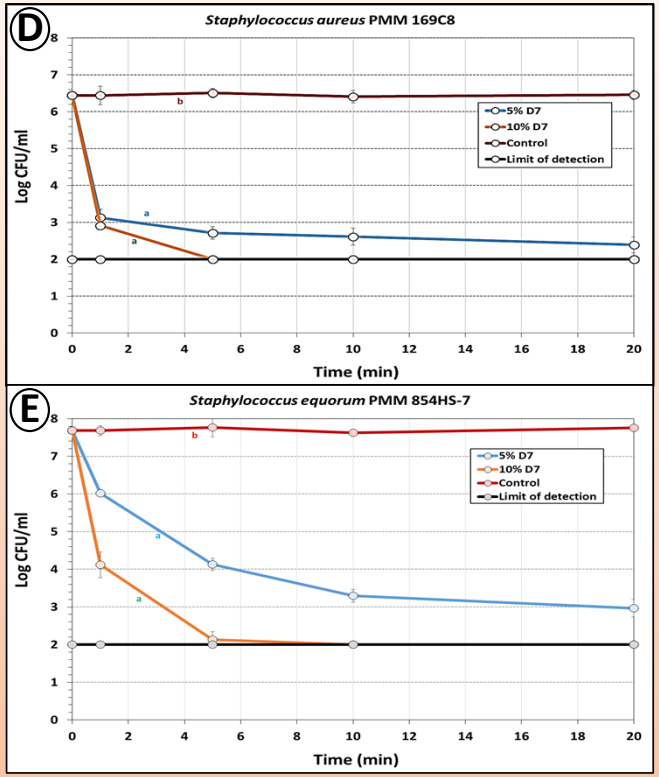


Figure 4. Biofilm microplate lethality assay on *Pseudomonas aeruginosa* 1 (panel A), *Pseudomonas aeruginosa* 2 (panel B), *Staphylococcus aureus* PMM 174C1 (panel C), *Staphylococcus aureus* PMM 169C8 (panel D), and *Staphylococcus equorum* PMM 854HS-7 (panel E) on 7-day biofilms challenged with 5% 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 data symbols). Treatments with different letters are significantly different (RM-ANOVA, $p < 0.05$); treatments with the same letters are not significantly different (RM-ANOVA, $p > 0.05$).

RESULTS (Cont.)

Treating Biofilms on Worker's Boots from the FAPC Slaughter Facility with Decon7. We could achieve ~3 log reduction in boots when sprayed with Decon7 solution combined with enzyme trypsin for subsequent detachment and enumeration. An average of 6 pairs of boots (in triplicate) were accessed to evaluate the sanitizer effectiveness against encrusted biofilms formed on the worker's boots. These generic biofilms develop on a regular basis when workers move into the slaughterhouse and each time bacteria accumulate, with nutrients and water, may enhance the formation of biofilm. Bi-Quat has been used in the slaughterhouse and apparently is not highly effective and may select for alkaliphilic organisms.

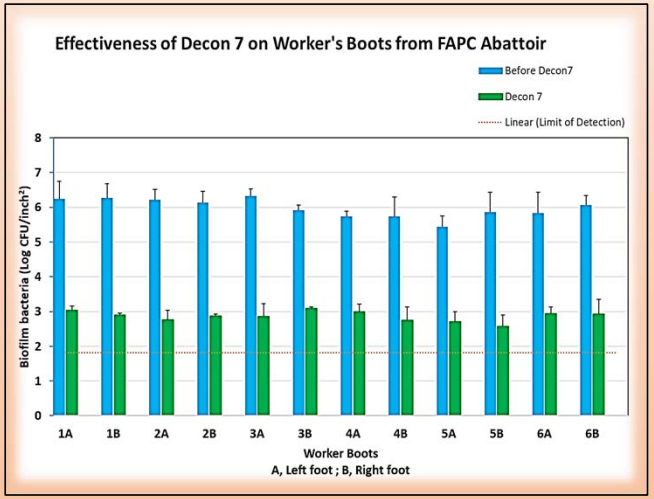


Figure 5. Enumeration of bacterial populations from encrusted biofilms in worker's boots. 'A' represents left foot and 'B' represents the right foot boot of one pair of boots. A total of 6 pairs of boots (3 replicate samplings of each) were assessed to isolate the bacterial strains and test the sanitizer lethality. Data points represent the means of replication and error bars represent the standard deviations from the means. The differences in the mean values among the treatment groups are greater than would be expected by chance; there is a statistically significant difference ($p < 0.05$).

RESULTS

DNA sequencing and sequence alignment. Seven (7) organisms isolated from boot biofilm were identified; *Pseudomonas parafulva, Pseudomonas lactis, Pantoea dispersa, Pantoea agglomerans, Aerococcus viridans, Bacillus velezensis and Enterococcus hirae*. These organisms are characterized based on the percentage identity obtained after 16S rRNA DNA sequence was matched with the sequence in the database. 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.

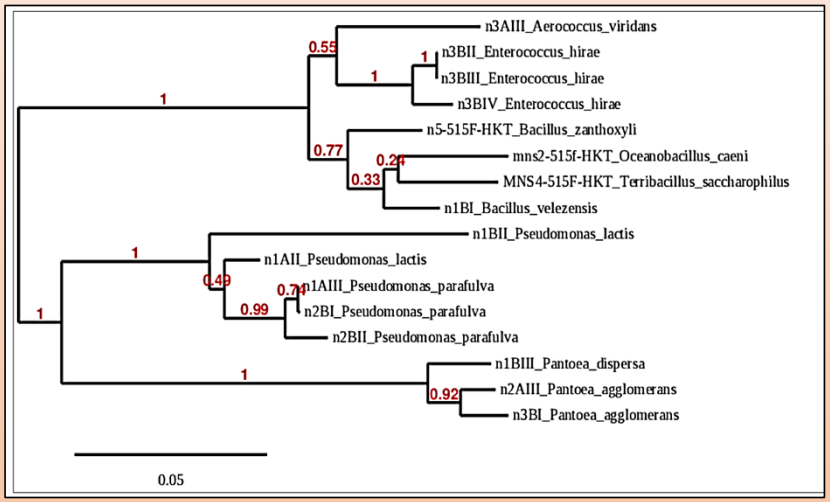


Figure 6. Phylogenetic tree of isolated bacterial strains from the encrusted biofilms on worker's boots, inferred by using maximum Likelihood method using bootstrap consensus tree. The value at the nodes represent bootstrap value (i.e. percentages based on 1000 replicates that signifies how many times out of 100, the same branch was observed when repeating the phylogenetic reconstruction on a re-sampled set of the data).

Antimicrobial Activity of Decon7 vs Bi-Quat against the Bacterial Isolates Derived from the Biofilms of Worker's Boots. The activity of Decon7 is greater than that of Bi-Quat. Additionally, the strains isolated from worker's boots showed less activity of the sanitizers as compared to the strains that were previously used as a lethality assay of Decon7.

RESULTS (Cont.)

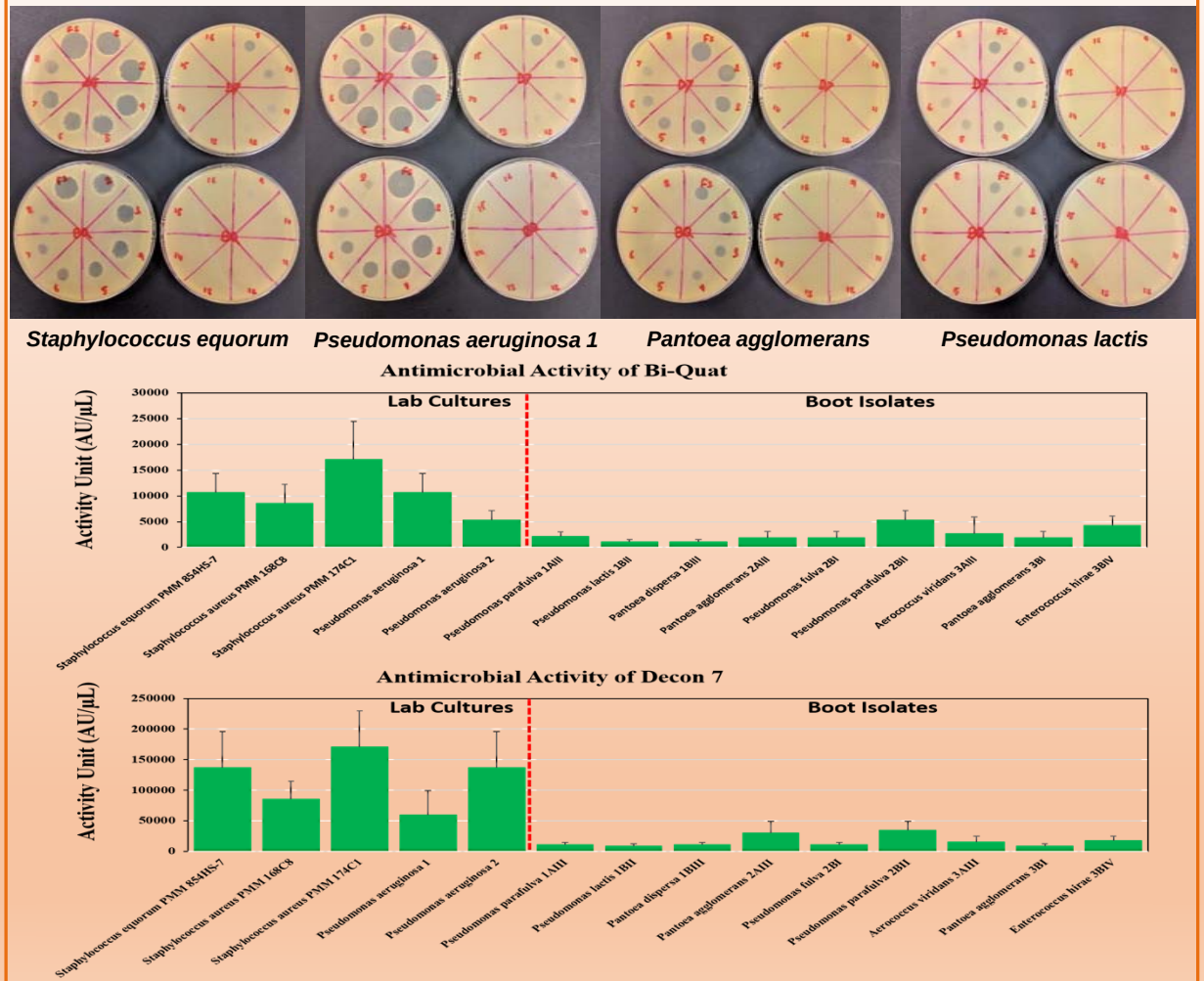


Figure 7. Antimicrobial activity of Bi-Quat and Decon7 against the bacterial strains used in lethality assay and bacterial isolates from the worker's boots (top plates). Comparison of antimicrobial activity of serial dilutions of Bi-Quat or Decon7 sanitizer spotted on lawns of laboratory bacteria (left side) vs isolates from boots (right side) to the sanitizer treatment.

DISCUSSION

The bacterial population in our laboratory biofilms achieved a 6.5-7.5-log CFU/ml level as determined by enzymatic detachment (likely diluted by recovery solution compared to attached CFU/cm^2 level). After treating the 7-day old biofilm with Decon7, we observed approximately ~4-5 log reduction in all strains tested within the first 1 min of treatment at 10% concentration.

The Decon7 that we used is a second generation QAC sanitizer, it includes hydrogen peroxide and Quaternary Ammonium Chloride along with diacetin as a booster.

Primarily, Decon7 is applied as a foam that provides greater interaction time on the surfaces in any facilities like an abattoir. Likewise QACs are found to be effective against both Gram-positive and Gram-negative bacteria. The surfactant acts on the phospholipid components in the cytoplasmic membrane that causes distortion and lysis, resulting in osmotic stress. Another component is hydrogen peroxide (H_2O_2) that acts as an oxidizer (7.9%) and 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 component and damages the cell.

Bi-Quat an early generation QAC has been used for a long time as a regular sanitizer in FAPC Meat Processing Pilot Plant. We used Decon7 on the boots of student worker's and could achieve ~3 log reduction. Regular use of Bi-Quat might have developed resistance in those boot isolates, thus providing less antimicrobial activity than with Decon7.

The microorganisms isolated were regularly subjected to the use of Bi-Quat which is an alkaline based sanitizer. Many of the isolated bacteria are known to be 'alkaliphillic' and can experience habituation to alkaline conditions, and this goes together with their increase in resistance to the sanitizer.

Plus, there is a high risk that the antimicrobial resistant microorganisms could transfer its resistant genes to the 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.

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CONCLUSION

In the course of conducting several studies (2 Master's student dissertations), we have found that Decon7 is an excellent commercial sanitizer that quickly and significantly reduces bacteria entrapped in biofilms such as *Listeria monocytogenes, Salmonella spp, E. coli* O157:H7, *Staphylococcus aureus,* and *Pseudomonas spp.*

Additional studies being planned to: We plan to use of Decon7 in an automated boot washer (and/or doorway foam system) to validate its effectiveness in practical applications in food processing situations.

Figure 8. Automated boot washer in the FAPC Slaughter Facility.

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