



Article

Efficacy of a Next Generation Quaternary Ammonium Chloride Sanitizer on *Staphylococcus* and *Pseudomonas* Biofilms and Practical Application in a Food Processing Environment

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Abstract: Foodborne pathogens are known to adhere strongly to surfaces and can form biofilms in food processing facilities; therefore, their potential to contaminate manufactured foods underscores the importance of sanitation. The objectives of this study were to (1) examine the efficacy of a new-generation sanitizer (Decon7) on *Staphylococcus* and *Pseudomonas* biofilms, (2) identify biofilm bacteria from workers' boots in relation to previous sanitizer chemistry, (3) validate the efficacy of Decon7 on biofilm from workers' boots from an abattoir/food processing environment, and (4) compare the sensitivity of isolated boot biofilm bacteria to new- and early (Bi-Quat)-generation QAC sanitizers. Decon7 was applied at two concentrations (5%, 10%) and was shown to be effective within 1 min of exposure against enhanced biofilms of *Staphylococcus* spp. and *Pseudomonas* spp. in 96-well microplates. Decon7 was also used to treat workers' boots that had accumulated high levels of biofilm bacteria due to ineffective sanitization. Bacteria isolated before enzyme/sanitizer treatment were identified through 16S rRNA PCR and DNA sequencing. All treatments were carried out in triplicate and analyzed by one-way RM-ANOVA or ANOVA using the Holm–Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$). The data show a significant difference between Decon7 sanitizer treatment and untreated control groups. There was a ~4–5 log reduction in *Staphylococcus* spp. and *Pseudomonas* spp. (microplate assay) within the first 1 min of treatment and also a > 3-log reduction in the bacterial population observed in the biofilms from workers' boots. The new next-generation QAC sanitizers are more effective than prior QAC sanitizers, and enzyme pre-treatment can facilitate biofilm sanitizer penetration on food contact surfaces. The rotation of sanitizer chemistries may prevent the selective retention of chemistry-tolerant microorganisms in processing facilities.

Keywords: sanitizer; quaternary ammonium chloride; QAC; QUAT; biofilm; *Staphylococcus*; *Pseudomonas*



Citation: Shah, K.; Muriana, P.M. Efficacy of a Next Generation Quaternary Ammonium Chloride Sanitizer on *Staphylococcus* and *Pseudomonas* Biofilms and Practical Application in a Food Processing Environment. *Appl. Microbiol.* **2021**, *1*, 89–103. <https://doi.org/10.3390/applmicrobiol1010008>

Academic Editor: Joaquín Bautista-Gallego

Received: 25 April 2021

Accepted: 24 May 2021

Published: 26 May 2021

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1. Introduction

Biofilms are a mixture of bacterial cells and organic material attached to surfaces [1]. Most biofilms evolve through a series of steps, involving initial microbial attachment, biofilm development and proliferation, followed by detachment and dispersal. Nearly any microorganism can initiate attachment to surfaces via Van der Waals interactions and electrostatic charges from numerous chemical constituents on its surface, including pili, fimbriae, flagella, lipopolysaccharides, and other proteins and carbohydrates [2]. The most important factor is the adherence provided by extracellular polysaccharides (EPS), which acts as a 'glue' for the biofilm matrix and may even trap other microorganisms that are not actively involved in biofilm formation [3,4]. Within a mature biofilm structure, different microenvironments may develop. The bacterial EPS may limit the transfer of nutrients and chemicals into the biofilm, which must diffuse through the 3-dimensional matrix to supply the embedded cells [5,6]. The disparity in the nutrient availability of cells on/near

the surface vs. those deeper inside the biofilm affects metabolism and genetic expression, resulting in heterogeneity even among the same type of cells in a biofilm [6,7].

Bacterial cells entrapped in biofilms are most readily subject to quorum sensing [8,9], a mechanism of genetic regulation often referred to as ‘cellular communication’ or, using older terminology, ‘bacterial pheromones’ [10]. The ability to self-regulate or regulate other cells that respond to the same autoinducer molecule is a matter of cell population density, whereby the released signal is capable of finding susceptible target cells, giving rise to the notion of requiring a ‘quorum’. There is likely no greater degree of high population density among bacteria than those growing on a surface (colony) or in a biofilm. Quorum sensing can be involved in many bacterial activities related to the microbial ecology of food and food manufacturing, including the promotion of adherence factors (attachment), antimicrobial resistance, the production of proteases (spoilage), or regulating the production of toxins and virulence factors by pathogenic bacteria [11–14]. For these reasons, good sanitation practices in food processing facilities are important and necessary.

Sanitation operations are generally two-fold, first requiring a cleaning regimen to remove large particles and aggregates of soiled organic material, followed by a disinfectant sanitation regimen to kill remaining bacteria [15]. Cleaning can be accomplished by simple physical means (scrubbing, sweeping), water spraying (low-pressure hoses, cleaning balls suspended into tanks, high-pressure nozzles), and more elaborate clean-in-place systems often found in sophisticated food processing equipment [16]. Cleaning is aided by the application of detergent along with hot water to soften hardened soils to facilitate their removal. A sanitizer is then used to disinfect the equipment once the large particle debris and organic material have been removed, allowing better access to any residual microbial contamination [16,17]. There are numerous categories of chemical sanitizers used for disinfection in food processing, from acidic to alkaline pH including chlorine-based oxidizers (hypochlorite, hypochlorous acid), non-chlorine oxidizers (ozone), peroxide-based oxidizers (hydrogen peroxide, peroxyacetic acid), iodophors, and quaternary ammonium compounds (Quat, QAC) [18]. Chlorine-based hypochlorite sanitizers are the most commonly used because of their ease of use and availability (i.e., bleach), however they are also limited in effectiveness when used at the maximum levels allowed for food contact surfaces (200 ppm). Given the municipal regulations against residual levels of chlorine in wastewater (USA) and the curtailed use of chlorine in other parts of the world (EU) because of the generation of chlorine-containing carcinogenic compounds, the use of chlorine is likely to be reduced in the future. The next most widely used chemical sanitizer is the family of QACs.

In this study we compared the effectiveness of a new QAC sanitizer (Decon7), representing a next-generation QAC sanitizer, on biofilms of *Staphylococcus* and *Pseudomonas* and compared the sensitivity of laboratory and biofilm-isolated bacteria to Decon7 and an early generation QAC sanitizer (Bi-Quat).

2. Materials and Methods

2.1. Bacterial Strains, Growth and Storage Conditions

Cultures were obtained from our laboratory culture collection (PMM#), and included *Pseudomonas aeruginosa* PMM 626 (P1), *Pseudomonas aeruginosa* PMM 627 (P2), *Staphylococcus aureus* PMM 174(C1), *Staphylococcus aureus* PMM 169(C8), *Staphylococcus equorum* PMM 854(HS-7), and other bacteria isolated, identified, and used during the course of this study (Table 1). These cultures were grown in tryptic soy broth (TSB, Bacto, BD, Franklin Lakes, NJ, USA) at 30 °C overnight and centrifuged (6000 × g, 5 °C) for 10 min. The supernatant liquid was discarded and the pellet was resuspended with a freezer storage medium (sterile TSB + 10% glycerol) and 4 mL of each cell suspension was transferred into 8-mL glass vials and stored at –80 °C. Frozen cultures were revived by thawing and transfer of 100 µL to 9 mL TSB and overnight incubation at 30 °C; cultures were sub-cultured twice before use. All assays were performed in triplicate with separate cultures grown for each replication, which were performed as independent experiments. Ten-fold serial dilutions

were performed with 0.1% buffered peptone water (BPW) and surface plated (100 μ L) on tryptic soy agar (TSA; TSB with 1.5% agar) in duplicate and incubated at 30 °C for 2 days before enumeration.

Table 1. Bacterial cultures used or isolated in this study.

Organism	Culture Collection Designation	Strain Designation	Source
<i>Pseudomonas aeruginosa</i>	PMM 626	P1	Isolated from processed egg facility
<i>Pseudomonas aeruginosa</i>	PMM 627	P2	Isolated from processed egg facility
<i>Staphylococcus aureus</i>	PMM 174	C1	Isolated from cow udders
<i>Staphylococcus aureus</i>	PMM 169	C8	Isolated from cow udders
<i>Staphylococcus equorum</i>	PMM 854	HS-7	Isolated from hotdogs
<i>Bacillus</i> sp.	PMM 435	MNS1	This study; abattoir worker boot
<i>Oceanobacillus</i> sp.	PMM 436	MNS2	This study; abattoir worker boot
<i>Terribacillus</i> sp.	PMM 437	MNS4	This study; abattoir worker boot
<i>Bacillus</i> sp.	PMM 438	MNS5	This study; abattoir worker boot
<i>Paenibacillus</i> sp.	PMM 439	MNS6	This study; abattoir worker boot
<i>Pseudomonas</i> sp.	PMM 440	KS1A2	This study; abattoir worker boot
<i>Pseudomonas</i> sp.	PMM 433	KS1A3	This study; abattoir worker boot
<i>Bacillus</i> sp.	PMM 441	KS1B1	This study; abattoir worker boot
<i>Pseudomonas</i> sp.	PMM 432	KS1B2	This study; abattoir worker boot
<i>Pantoea</i> sp.	PMM 430	KS1B3	This study; abattoir worker boot
<i>Pantoea</i> sp.	PMM 428	KS2A3	This study; abattoir worker boot
<i>Pseudomonas</i> sp.	PMM 431	KS2B1	This study; abattoir worker boot
<i>Pseudomonas</i> sp.	PMM 434	KS2B2	This study; abattoir worker boot
<i>Aerococcus</i> sp.	PMM 426	KS3A3	This study; abattoir worker boot
<i>Pantoea</i> sp.	PMM 429	KS3B1	This study; abattoir worker boot
<i>Enterococcus</i> sp. (<i>hirae</i>)	PMM 442	KS3B2	This study; abattoir worker boot
<i>Enterococcus</i> sp. (<i>hirae</i>)	PMM 443	KS3B3	This study; abattoir worker boot
<i>Enterococcus</i> sp.	PMM 427	KS3B4	This study; abattoir worker boot

2.2. Growth of Enhanced Biofilms in Microplates

Cultures of *Staphylococcus* sp. and *Pseudomonas* sp. were grown overnight at 30 °C (~9 log CFU/mL) and diluted to ~4 log CFU/mL in TSB. Falcon 96-well, clear, flat-bottom microplates (Cat# 351172, Corning, NY, USA) were used as the substrate to create biofilms. The microplate biofilm procedure was similar to those used previously in our lab [19–21]. Prior to using a particular type of microplate to create biofilms for this study, we examined both cell-culture-treated plates and untreated plates. A 200- μ L aliquot of the TSB-diluted culture was allocated into microplates, which were sealed with Parafilm (Fisher Scientific, Atlanta, GA, USA) to avoid evaporation and incubated for 24 h at 30 °C. The microplates were then washed three times with sterile Tris buffer (pH 7.4, 50 mM) in a Biotek Elx405 Magna plate washer (Ipswich, Suffolk, UK) using the ‘shake’ option to release loosely held cells, followed by the addition of 200 μ L of fresh sterile TSB. The microplates were then sealed with Parafilm and incubated for 24 h at 30 °C. This cycle of growing, washing, and media renewal was repeated daily for 7 days to develop robust biofilms. The Biotek Elx405 Magna plate washer was used to wash the microplates to remove planktonic and loosely held cells from the biofilm; the fresh media replacement was to allow enrichment of the adhered cells of the biofilm layer. The plate washer was connected to different wash solutions depending on the need: 10% bleach solution (to sanitize the lines), sterile de-ionized water (to flush out the bleach), sterile 50 mM Tris buffer (pH 7.4; to wash microplates), and waste containers for the residual wash effluent. Before use with microplates, the plate washer needles were sanitized by running a maintenance cycle using 10% bleach (2 \times times), sterile de-ionized water (3 \times times), and sterile Tris buffer (2 \times times).

After 7 daily cycles of washing the biofilms, addition of fresh sterile media, and further incubation, the final wash was carried out with Tris buffer. A trypsin enzyme solution was used for the detachment of adhered cells from the microplates to obtain a plate count enumeration from the biofilm, as described previously [19–21]. Trypsin solution (Cat: T4549; Sigma-Aldrich, St. Louis, MO, USA) was diluted with sterile phosphate-buffered saline (137 mM NaCl, pH 7.4; Santa Cruz Biotechnology, Santa Cruz, CA, USA) in the ratio 1:3 (~500 U/mL) and transferred into the experimental wells, sealed with Parafilm, and

incubated at 37 °C for 1 h. The solutions from the wells were diluted (i.e., 200 µL of well cell suspension was transferred to 1800 µL of Tris buffer solution, followed by 1-mL 10-fold serial dilutions), plated on TSA, and incubated for 24–48 h at 30 °C for enumeration.

2.3. Microplate Biofilm Sanitizer Assay

Decon7 sanitizer solution (Decon™ Seven Systems, Scottsdale, AZ, USA) and Bi-Quat (#I00254, Birko, Henderson, CO, USA) were the sanitizers used in our studies.

Bi-Quat is a 10% dual n-alkyl ammonium chloride (5% n-alkyl dimethyl benzyl and 5% n-alkyl ethyl benzyl ammonium chloride) disinfectant used at up to 1000 ppm on environmental surfaces and 200 ppm for food contact surfaces. Bi-Quat has been used continuously in the R. M. Kerr Food and Products Center (FAPC) abattoir for 20+ years to sanitize equipment and workers' boots.

Decon7 is a 3-component disinfectant that is supplied as 3 solutions: part 1, a surfactant (quaternary ammonium compound; benzyl and dimethyl chlorides (5.5–6.5%); part 2, an oxidizer (hydrogen peroxide (7.9%); and part 3, an accelerator (diacetyl). These three parts were mixed in the ratio 2:2:1, according to the manufacturer's instructions, to form the concentrated stock solution. Working stock solutions were made at 5% and 10% concentrations of the concentrated stock solution to assess efficacy against biofilms. As an assessment of the bactericidal ability of Decon7, both *St. aureus* PMM 174 and *Ps. aeruginosa* PMM 626 cultures were added directly to Decon7 (5%) and to BPW, recovered in D/E broth, pour plated (1 mL) in TSA, and incubated at 30 °C for 48 h for the enumeration of residual viable cells.

Different microplates were used for each Bi-Quat and Decon7 application time of 1, 5, 10, and 20 min. After 7 daily cycles of growing biofilm, microplates were washed 3× with sterile Tris buffer (50 mM, pH 7.4). Treatments with Bi-Quat and Decon7 were then applied to the various biofilms created with 5 different strains (2 strains of *Pseudomonas aeruginosa* and 3 strains of *Staphylococcus* sp.). Decon7 (200 µL, 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 (50 mM, 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/Bi-Quat residue [22,23]. After treatment for 5 min with D/E neutralizing broth, microplates were washed with Tris buffer (50 mM, pH 7.4). Trypsin solution (200 µL; ~500 U/mL) was then added into the wells of microplates for the enzymatic detachment of attached/residual viable bacterial cells. Microplates were sealed with Parafilm, incubated at 37 °C for one hour, plated on TSA plates in duplicate, and incubated at 30 °C for 48 h and enumerated. Microbial populations were converted to log denomination and plotted vs. time of treatment.

2.4. Enumerating Biofilm Levels from Workers' Boots

To determine the indigenous level of bacteria in boot biofilms, a Sponge-Stick™ with neutralizing buffer (3M, Saint Paul, MN, USA) was used to swab the biofilm present on the 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 were swabbed in different marked areas for each of 3 replicate samplings for both left and right boots of a pair, and both before and after sanitizer application. The swab stick was turned over to adsorb more cells and then the sponge was pulled off into a sampling bag. The sampling bag plus the sponge was stomached and plated on TSA plates. Bacteria isolated from workers' boots were identified by means of 16S rRNA gene PCR and DNA sequencing. The effectiveness of Decon7 on the boot biofilm was examined using a liquid spray containing 10% Decon7 stock solution and sprayed manually on the boots (~14 sprays/boot; ~18 mL). After 5 min, the boots were rinsed with water and swabbed with a sponge stick with neutralizing buffer and trypsin solution. The sponge stick was stomached, serially diluted in 0.1% BPW, and plated on TSA in duplicate, incubated at 30 °C, and enumerated after 48 h.

2.5. Identifying Biofilm Bacteria from Workers' Boots: 16S rRNA PCR and Sequencing

Bacterial colonies obtained from the enumeration of bacteria from workers' boots representing the highest bacterial counts (i.e., the highest dilution) were re-streaked, isolated, and stored in our culture collection, as described above.

The identities of 16S rRNA DNA sequences were determined as described previously [24,25]; recommended primers were derived from published sources [26,27]. Bacterial cultures were grown overnight in TSB at 30 °C and 1 mL was pelleted in 1.5-mL Eppendorf tubes by means of centrifugation (12,000× g, 1 min). The supernatant was discarded and cells were suspended by adding 0.5 mL of Tris buffer (50 mM, pH 7.4) followed by centrifugation (repeated 3×). After a third pelleting, the cell pellet was suspended with 200 µL of Tris buffer (50 mM, pH 7.4). Nitric acid-washed glass beads (Fisher Scientific) were added with a small sterile spatula to the conical tube with the resuspended cell pellet, halfway up the liquid portion. This was vortexed for 2 min in an Eppendorf tube-holder adapter (i.e., 'bead-beating'), followed by holding for 4 min on ice (repeated 3×) [28]. This was further centrifuged at 12,000× g for 2 min to recover DNA by removing 50–100 µL of the supernatant solution above the glass beads into a new sterile tube.

The extracted DNA was quantified using a Nanodrop 1000 spectrophotometer (ND-1000, Thermo Scientific, Waltham, MA, USA) by measuring the nucleic acid present. The extracted DNA was diluted with nuclease-free water to a working concentration of ~1 ng/µL. For PCR amplification, the sample was prepared by adding 5 µL (5 ng) of DNA template, 12.5 µL of Promega Master Mix (Promega, Madison, WI, USA), 1 µL each of forward (515-F; 5'-GTGCCAGCMGCCGCGGTAA-3') and reverse (1391-R; 5'-GACGGGCGGTGWGTRCA-3') primers (0.5 µM), and 5.5 µL of nuclease-free water. The samples were loaded into PCR microplates in an Opticon 2 thermocycler (MJ Research, Bio-Rad Laboratories, Hercules, CA, USA). The PCR parameters included an initial denaturation (95 °C, 4 min), followed by 40 cycles of denaturation (94 °C, 30 s), annealing (50 °C, 30 s), and extension (72 °C, 60 s), and a final hold at 4 °C until retrieval. The PCR-amplified product was purified from the remaining PCR enzyme, dNTPs, and salts using the Epoch GenCatch™ PCR Cleanup Kit (Epoch Life Sciences, Sugarland, TX, USA) and then submitted to the OSU DNA Core Facility for DNA sequencing. Mega-X software (<https://megasoftware.net/>, accessed on 1 May 2021), a cross-platform free shareware program, was used for the sequence alignment and inference of phylogenetic trees of bacterial partial 16S rRNA DNA sequences obtained from workers' boots [29]. The maximum likelihood method was used for analysis of phylogeny using the bootstrap method.

2.6. Comparison of New-Generation QAC (Decon7) with Early-Generation QAC (Bi-Quat) Sanitizer

Bi-Quat, an early-generation QAC, has been used in the FAPC abattoir and food processing facility as a sanitizing agent for over 20 years. One of our objectives was to compare the antimicrobial activity of Bi-Quat and Decon7 on the *Staphylococcus* sp. and *Pseudomonas* sp. used in this work and on the bacterial isolates recovered from workers' boots. A soft agar overlay technique was used to screen the effect of Bi-Quat and Decon7 sanitizers against bacterial lawns of these strains. The 'spot-on-lawn' method is a semi-quantitative method to identify the sensitivity of strains to serial dilutions of antimicrobial substances [30,31].

Each bacterial strain was grown overnight in TSB at 30 °C and 50 µL of culture was added to 5 mL of soft agar TSA (0.75% agar). The TSA/soft agar with inoculated culture was poured on top of pre-poured TSA plates (1.5% agar) 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. Both sanitizers were serially diluted by transferring 100 µL of full-strength solution (working stock) from one well to 100 µL of sterile water into another well; this carryover was continued for up to sixteen 2-fold dilutions. Soft agar overlay plates were divided into 16 pie sections over two Petri plates (8 sections/plate) to test sanitizer activity against the bacterial-seeded overlays. The

sanitizer dilutions (5 µL) were spotted over each section and allowed to sit for 10 min before being incubated for 24 h at 30 °C. Antimicrobial activity was then observed for each strain against the two sanitizers and enumeration was based on the last serial dilution showing inhibitory activity. Inhibitory activity is based on the reciprocal of the last serial dilution showing inhibitory activity and indicated as 'activity units' (AUs) per milliliter. The same antimicrobial showing different AU levels on various strains would be representative of the sensitivity (higher AUs) or resistance (lower AUs) of those strains to the antimicrobial.

2.7. Statistical Analysis

Each trial was performed in triplicate as separate and independent experiments, using separate cultures and prepared media. The statistics functions in the software package SigmaPlot ver. 13 (Systat Software, San Jose, CA, USA) were used for statistical analyses performed in this study. The effect of sanitizers on biofilms over different times (time-series plots) was statistically analyzed using repeated-measures one-way analysis of variance (RM-ANOVA) with the Holm–Sidak test for multiple pairwise comparisons. Repeated-measures analysis involves the statistical comparison of an entire treatment data set to another complete set of treatment data with significance ($p = 0.05$) relative to the entire data set (curves). Bacterial sensitivity to sanitizers was statistically analyzed using one-way ANOVA with the Holm–Sidak method for pairwise multiple comparison procedures. The data displayed are the average of triplicate replications and error bars represent the standard deviation of the mean. Data with different letters are significantly different ($p < 0.05$); treatments with the same letter are not significantly different ($p > 0.05$).

3. Results and Discussion

3.1. Generating Microplate Biofilms of Strains of *Staphylococcus* and *Pseudomonas*

In our prior work, untreated microplates were used to examine the innate ability of strains of *Listeria monocytogenes* isolated from food processing plants to form biofilms [19,32,33]. In the current study, treated microplates were used to facilitate as high a degree of adherence as possible to evaluate sanitizer effectiveness on biofilms. We used cell-culture-treated flat-bottom 96-well microplates and repeated daily cycles of washing and fresh media supplementation to create 7-day enhanced biofilms of *Ps. aeruginosa* PMM 626, *Ps. aeruginosa* PMM 627, *St. aureus* PMM 171, *St. aureus* PMM 169, and *St. equorum* PMM 854. In the US, the Environmental Protection Agency (EPA) regulates the use of pesticides, and sanitizer effectiveness against biofilms is evaluated with *Staphylococcus* sp. and *Pseudomonas* sp. [28]. The use of treated cell culture plates facilitated the adherence of bacteria to the wells and produced an increase of 0.74–1.44 log in biofilm development compared to when using untreated plates, and therefore treated plates were used for the remainder of the study (Figure 1).

3.2. Efficacy of Decon7 on *Staphylococcus* sp. and *Pseudomonas* sp. Biofilms

Decon7 is a new-generation QAC sanitizer and consists of a three-part solution formulation: a surfactant (quaternary ammonium compound), an oxidizer (hydrogen peroxide), and an accelerator (diacetyl). Decon7 was used in lethality assays on biofilms generated with two strains of *Pseudomonas* sp. (*Ps. aeruginosa* PMM 626, *Ps. aeruginosa* PMM 627) and three strains of *Staphylococcus* sp. (*St. aureus* PMM 174, *St. aureus* PMM 169, *St. equorum* PMM 854) at both 5% and 10% concentrations of the stock solution.

Decon7 demonstrated excellent reductions against *Staphylococcus* sp. and *Pseudomonas* sp., as was previously observed against *L. monocytogenes*, *Salmonella* Montevideo, and *E. coli* O157:H7 [20]. Decon7 was able to provide 4.5–5.7 log reductions of *Staphylococcus* strains (Figure 2). Both 5% and 10% Decon7 showed no significant difference in treatments with either *St. aureus* strain; however, *St. equorum* showed significantly different efficacies with 5% and 10% Decon7 (Figure 2). Similarly, 5% Decon7 showed a 2.6–4.1 log reduction, whereas 10% Decon7 demonstrated a 3.2–5.1 log reduction, respectively, of *Pseudomonas* sp. (Figure 3).

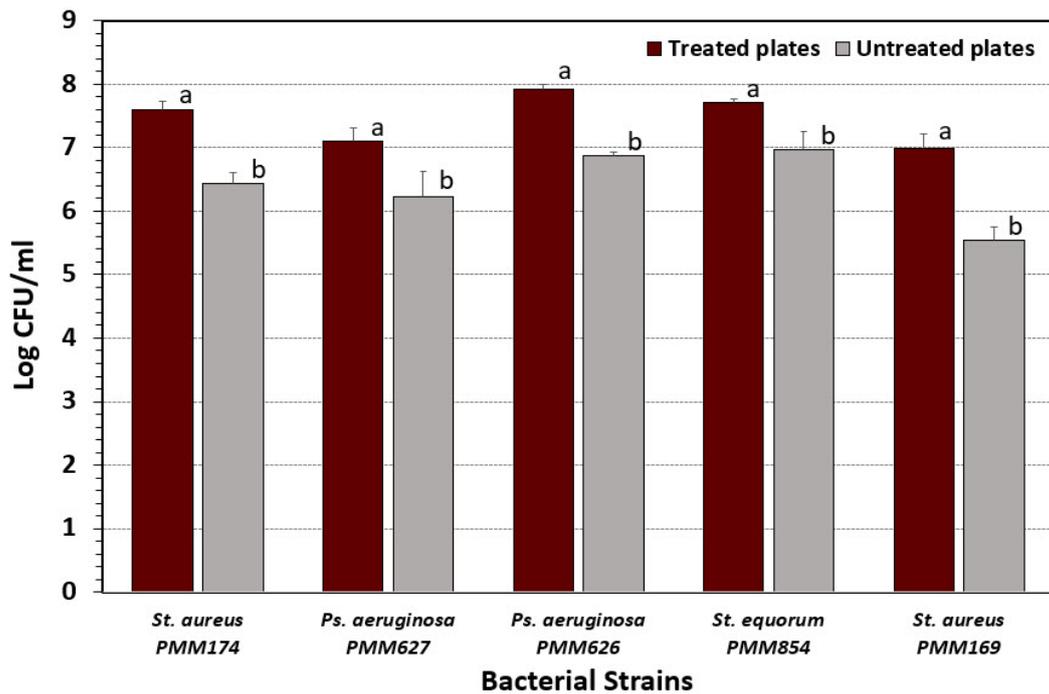


Figure 1. Comparison between biofilm levels obtained with various *Staphylococcus* and *Pseudomonas* strains used in this study on 96-well cell-culture-treated plates and untreated plates. All data are presented as the means of triplicate replications; error bars represent the standard deviation from the mean. Statistical analysis was performed using one-way analysis of variance (ANOVA), using the Holm–Sidak method of comparison to determine the significance of data from treated/untreated places for a given strain. Data bars with the same letter are not significantly different ($p > 0.05$); data bars with different letters are significantly different ($p < 0.05$).

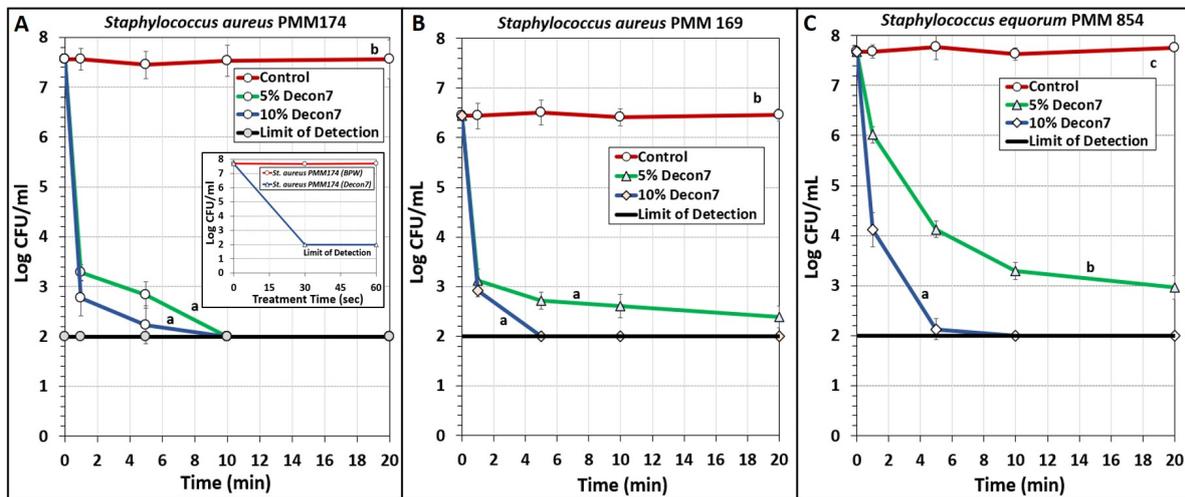


Figure 2. Biofilm lethality assays with 5% and 10% solutions of Decon7 vs. biofilms generated by strains of *Staphylococcus* sp. in cell-culture-treated microplates: (A) *St. aureus* PMM 174; (B) *St. aureus* PMM 169; (C) *St. equorum* PMM 854. Controls were from biofilms treated with 0.1% BPW. The embedded graph (Panel A) represents the viability of cells added directly to Decon7 solution vs. buffered peptone water (BPW). All data are presented as the means of triplicate replications; error bars represent the standard deviation from the means. Statistical analysis was performed using repeated-measures one-way analysis of variance (RM-ANOVA), using the Holm–Sidak method of comparison to determine the significance of treatment comparisons with a given strain. Time-course data sets with the same letter are not significantly different ($p > 0.05$); data sets with different letters are significantly different ($p < 0.05$).

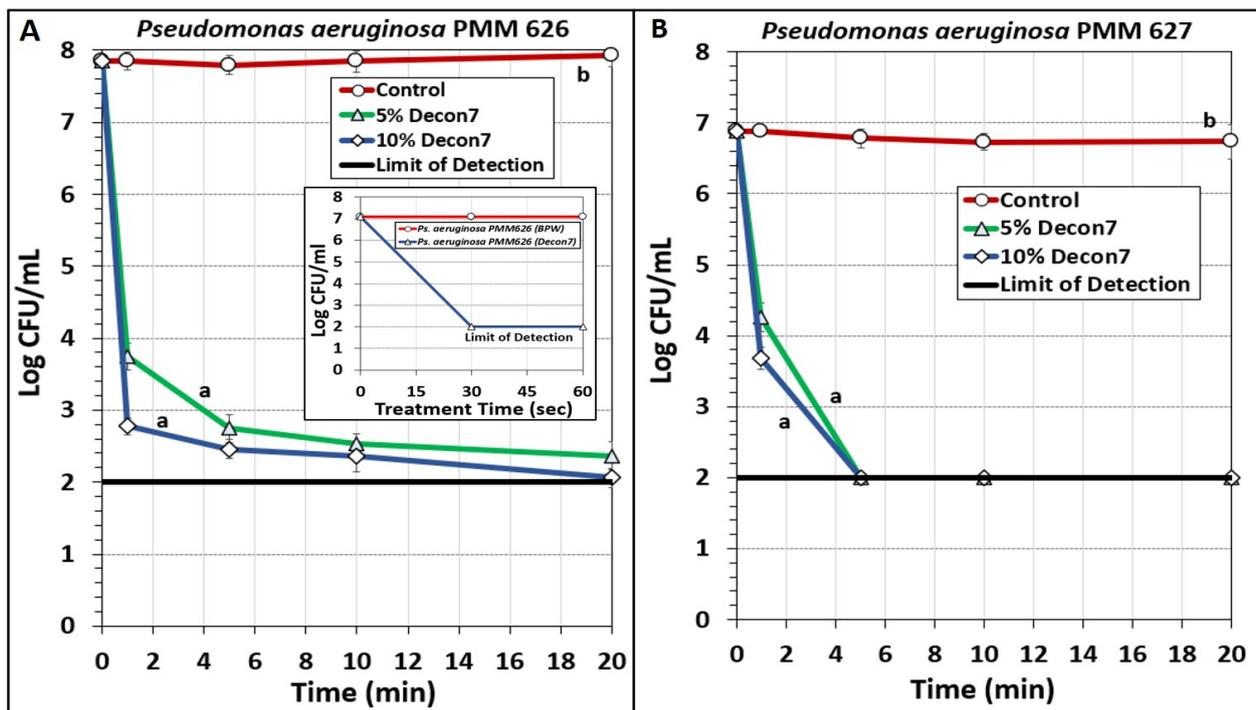


Figure 3. Biofilm lethality assays with 5% and 10% solutions of Decon7 vs. biofilms generated by strains of *Pseudomonas* sp. in cell-culture-treated microplates: (A) *Ps. aeruginosa* PMM; (B) *Ps. aeruginosa* PMM. The embedded graph (Panel A) represents the viability of cells added directly to Decon7 solution vs. buffered peptone water (BPW). Controls were from biofilms treated with 0.1% BPW. All data are presented as the means of triplicate replications; error bars represent the standard deviation from the means. Statistical analysis was performed using repeated-measures one-way analysis of variance (RM-ANOVA), using the Holm–Sidak method of comparison to determine the significance of treatment comparisons with a given strain. Time-course data sets with the same letter are not significantly different ($p > 0.05$); data sets with different letters are significantly different ($p < 0.05$).

Although we introduced triple shake-washing procedures in the washing step of our biofilms to stabilize and minimize the bacterial release from the microplate biofilms [20,21], there could still be some bacterial release during treatment with sanitizer solutions. We briefly examined the viability of both *St. aureus* PMM 174 (inset on Figure 2A) and *Ps. aeruginosa* PMM 626 (inset on Figure 3A) added directly to Decon7 and showed that both were immediately reduced by >7-logs within 30 s. These data suggest that biofilm bacterial cells released during sanitizer treatments would be more readily killed than those embedded in the biofilms during the same treatment time. This is similar to what we observed previously with hypochlorous acid and three different pathogens [34].

3.3. Efficacy of Decon7 on Natural Biofilms on Workers' Boots from a Food Processing Abattoir

The workers' boots from the FAPC abattoir showed a high level of bacterial biofilm (~6 log CFU/inch²) when swabbed with trypsin solution using a sponge stick and enumerated on TSA plates (Figure 4). We observed a ~3-log reduction when boots were sprayed with Decon 7 solution and swabbed with trypsin/neutralizing broth for enumeration (Figure 4). Six pairs of boots with three replicates each, before and after Decon7 sanitizer treatment, were evaluated for the sanitizers' effectiveness against natural biofilms found on workers' boots. These generic biofilms develop on a regular basis when workers move into the abattoir and whenever the boots come into contact with nutrients (animal tissues, blood) and water, which enhances their formation. Bi-Quat and hot water application has been used for >20 years as a generalized boot sanitation scheme and, like many such sanitation regimens in busy food facilities, there is often little or no verification of their efficacy.

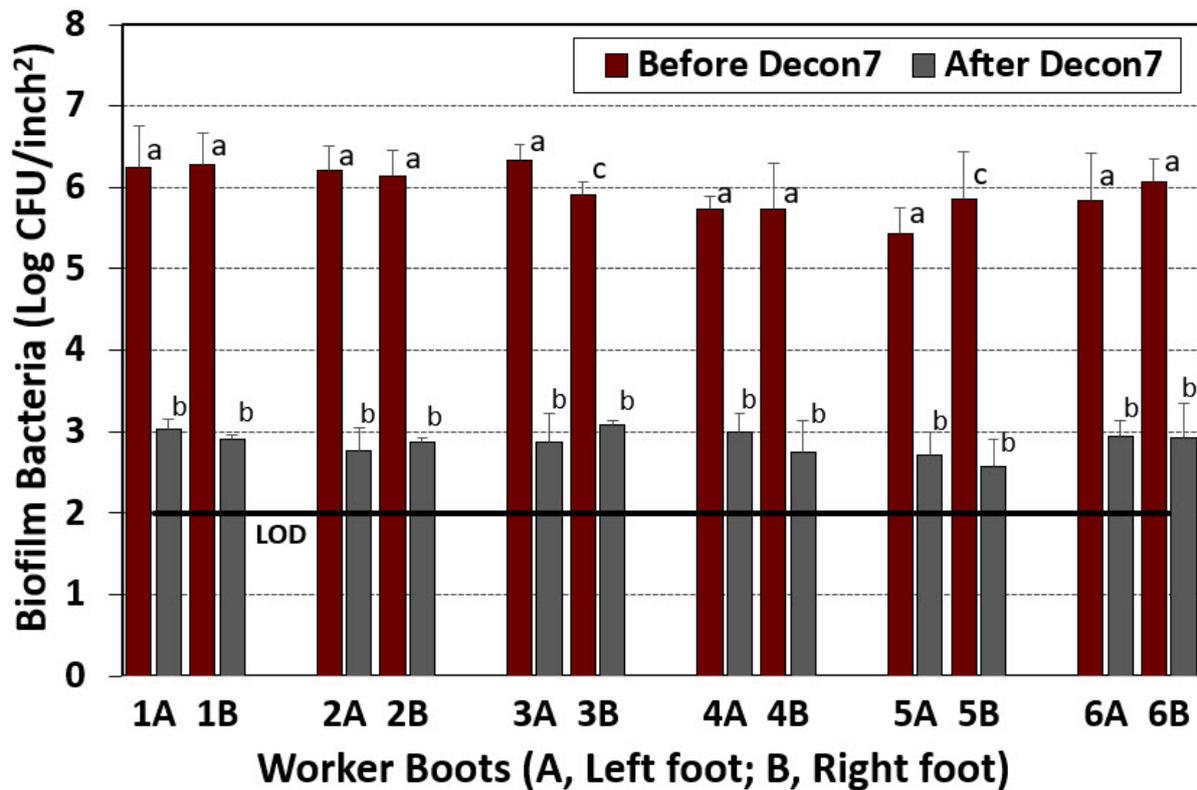


Figure 4. Recovery of bacteria from 6 pairs of workers' boots from the FAPC abattoir before and after the application of Decon7. Data are presented as the means of triplicate replications, and error bars represent the standard deviation from the mean. Comparisons were only performed within the same pair of boots (A and B); means with different letters are significantly different ($p < 0.05$) as determined by one-way ANOVA, using the Holm–Sidak test for pairwise multiple comparisons; means with the same letter are not significantly different ($p > 0.05$). Limit of detection, LOD.

Biofilms develop in a stepwise process, beginning with the attachment of individual cells (reversible), followed by secretion of polymeric substances, which helps in the binding of cells more firmly in a heterogeneous way. Once a mature biofilm develops, it is irreversible and difficult to remove in commercial facilities. It is often the focus of contamination, whereby cells are dispersed to contaminate new locations or even food that is being processed. Staphylococci are known to be prodigious producers of biofilms [35]. Numerous genes have been identified in *St. aureus* that are involved with the formation of biofilms, including the intercellular adhesion (*ica*) gene complex, which was found to be actively expressed in numerous biofilm isolates from the food industry [36]. These genes are involved in the biosynthesis of an extracellular polysaccharide matrix that protects the entrapped bacterial population from unfavorable environmental conditions (i.e., sanitizers). In another study of 97 isolates obtained from food samples, 72% of the strains were able to make biofilms and all possessed genes for the intercellular adhesion locus (*icaADBC*) and fibrinogen-binding clumping factors (*clfA/B*, *fib* genes), but none had genes for biofilm-associated protein (*bap*) [37]. In our study, developing a robust biofilm of each bacterial strain was mediated by means of continuous washing with Tris buffer to remove planktonic and loosely adhered cells, and by adding fresh nutrient medium each day for 7 days. The bacterial population in our biofilm achieved ~6.5–7.8-log CFU/mL upon proteolytic detachment and recovery from the biofilm. After treating the 7-day old biofilm with Decon7, we observed an approximately ~3–5 log reduction in all the strains tested in microplate assays within 1 min of treatment using the 10% concentration (Figures 2 and 3).

3.4. Recovery and Identification of Bacteria from Biofilms from Workers' Boots in a Food Processing Abattoir

We used 16S rRNA PCR to identify the organisms present on the biofilms of workers' boots. Eighteen organisms were isolated and characterized, namely, *Pseudomonas* sp. (×5), *Pantoea* sp. (×3), *Aerococcus* sp. (×1), *Enterococcus* sp. (×3), *Bacillus* sp. (×3), *Oceanobacillus* sp. (×1), *Terribacillus* (×1), and *Paenibacillus* sp. (×1) (Table 1). These organisms are characterized based on the percentage identity obtained when the sequences after DNA sequencing were subjected to a nucleotide BLAST search and matched with DNA sequences in the NCBI database [38,39]. It is generally accepted by most taxonomists that a 'percent identity' score of $\geq 97\%$ and $\geq 99\%$ for a 16S rRNA gene sequence is appropriate to identify an organism down to the genus or species level, respectively [39–41]. However, using a partial 16S rRNA gene sequence, when multiple species are identified with the same degree of sequence identity matching, bacterial identity was limited to the genus-level (Figure 5). The phylogenetic tree was inferred using maximum composite likelihood as a statistical method and the bootstrap method was used as the test of phylogeny, with the bootstrap number of replications being 1000 (Figure 5).

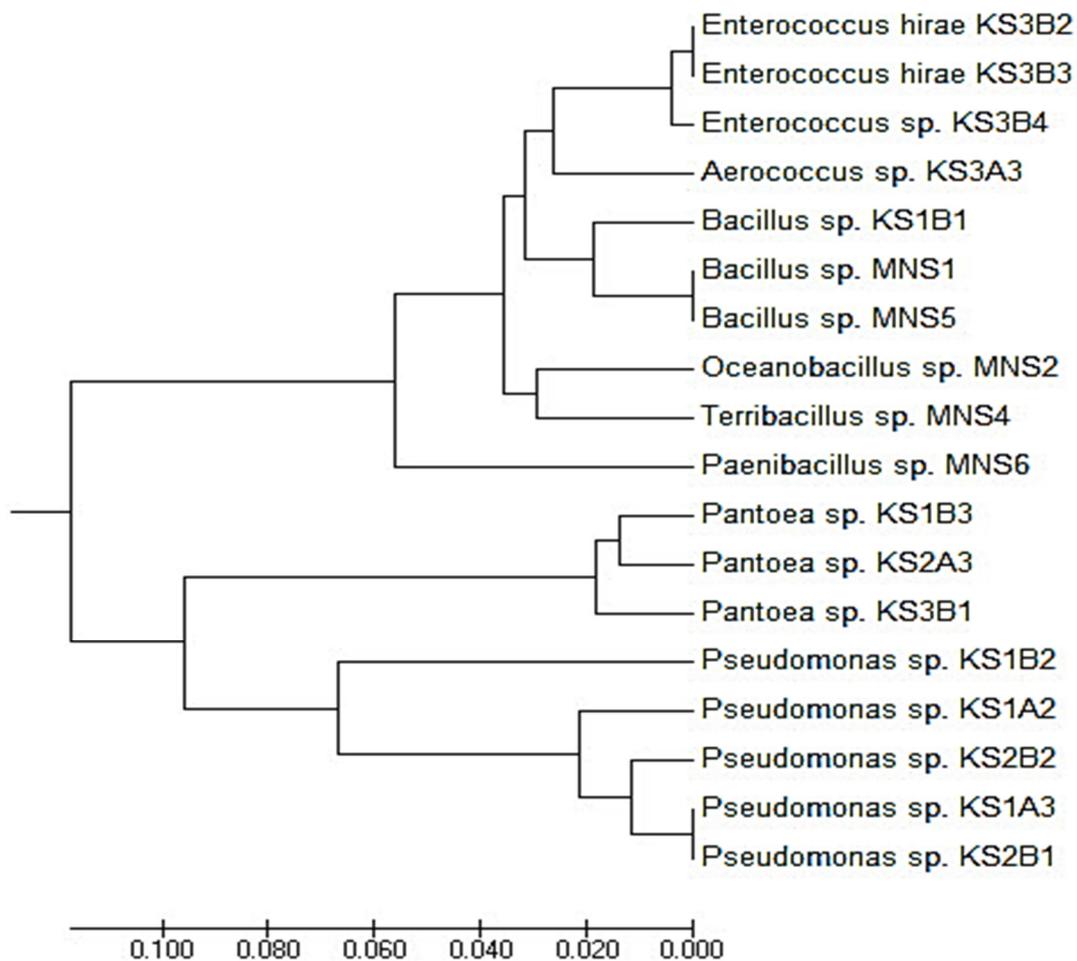


Figure 5. The phylogenetic relatedness of the boot biofilm bacteria isolated in our study. The relatedness was inferred using the UPGMA method. The evolutionary distances were computed using the maximum composite likelihood method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA7.

3.5. Sensitivity of Bacteria from Biofilm from Workers' Boots to Dual-Quat (Bi-Quat) vs. Quad-Quat (Decon7) Sanitizers

Decon7 is a next-generation QAC sanitizer that incorporates two different modes of action, including hydrogen peroxide and quaternary ammonium chloride, along with diacetin as a surfactant booster. It has been shown to be effective against biofilms formed with traditional foodborne pathogens such as *E. coli* O157:H7, *Listeria monocytogenes*, and various *Salmonella* serovars [42,43]. QACs are cationic surfactants that have a cleaning and cell-disruptive activity associated with them. They are effective against both Gram-positive and Gram-negative bacteria, whereby they interact with phospholipid components in the cell membrane, causing distortion and osmotic stress [44]. Diacetin is an ester of glycerol and acetic acid (glycerol diacetate) that facilitates the surfactant interaction of QACs. The hydrogen peroxide (H₂O₂) in Decon7 provides a different mode of action, acting as an oxidizer. It is a broad-spectrum sanitizer on its own and is innocuous to the environment. As an oxidant, it releases hydroxyl free radicals (•OH) that are unstable and act on lipids, proteins and DNA, specifically targeting the double bonds and sulfhydryl groups within the cell wall component and damaging the cell [45].

Bi-Quat is an early generation QAC that has been used for >20 years as a regularly used sanitizer in the FAPC Meat Processing Pilot Plant. High levels of biofilm bacteria were found on all boots tested from student workers in the FAPC slaughterhouse facility, suggesting that sanitation practices were not effective on workers' boots. When Decon7 was used on student workers' boots, a ~3-log reduction was achieved (Figure 4). The regular use of Bi-Quat (single mode of action) might have allowed the accumulation of sanitizer-tolerant bacteria in the boot biofilm, explaining the reduced sensitivity of boot biofilm isolates in the antimicrobial spot assays with both Decon7/Bi-Quat (Figure 6). This phenomenon has been observed elsewhere [46,47] and some bacterial genera such as *Staphylococcus* sp. [48,49] and *Pseudomonas* sp. [50] have even been shown to harbor plasmids that provide resistance to QACs.

As cationic surfactants, QACs have found many uses among industrial and domestic applications as emulsifiers, fabric detergents, surfactants, and disinfectants for hospitals and food manufacturing facilities. Many of the bacteria isolated from boot biofilms in this study (Table 1) are known to be alkaline-tolerant, including *Pantoea* sp. [51], *Oceanobacillus* sp. [52], *Paenibacillus* sp. [53], and *Terribacillus* sp. [54]. *Enterococcus* sp. (*hirae*) is also alkaline resistant and has been used to ferment molasses to lactic acid when grown under alkaline conditions [55,56]. Investigations on the persistence of human pathogens in healthcare facilities, where QAC disinfectants are commonly used, have identified a series of *qac*-genes involved in the membrane export of QACs as a mechanism of resistance in *Enterococcus* sp., *Staphylococcus* sp., and *Pseudomonas* sp. [57]. Similarly, some *Bacillus* sp. are not only resistant to alkaline solutions, but can also degrade QACs [58]. Although we isolated *Bacillus* sp. and other spore-forming bacteria among our boot biofilm isolates (Table 1), it is not clear if this was the result of the recovery of vegetative cells or spores that later germinated during isolation, as spores are known to be more alkaline-resistant than vegetative cells [59]. Isolates of *Pseudomonas* sp. from boot biofilm bacteria (Table 1) represent the largest clade of our taxonomic dendrogram (Figure 5) and are also associated with alkaline QAC resistance [60,61].

The cultures used in this study were examined for sensitivity to Decon7 and Bi-Quat in spot-on-lawn assays (Figure 6). The data showed that all the strains were more sensitive to Decon7 than to Bi-Quat, indicating that Decon7 would be much more effective as a disinfectant, as demonstrated previously with laboratory-generated biofilms [43] and in this study with additional laboratory (Figures 2 and 3) and natural biofilms (Figure 4). The alkaline-tolerant bacteria recovered from boot biofilm also show an increased resistance to both Decon7 and Bi-Quat compared to the laboratory strains. These data confirm that the constant use of a singular mode-of-action sanitizer at sublethal levels provides a selective condition that may result in the accumulation of resistant organisms that in some cases may even promote cross-resistance to antibiotics [62,63].

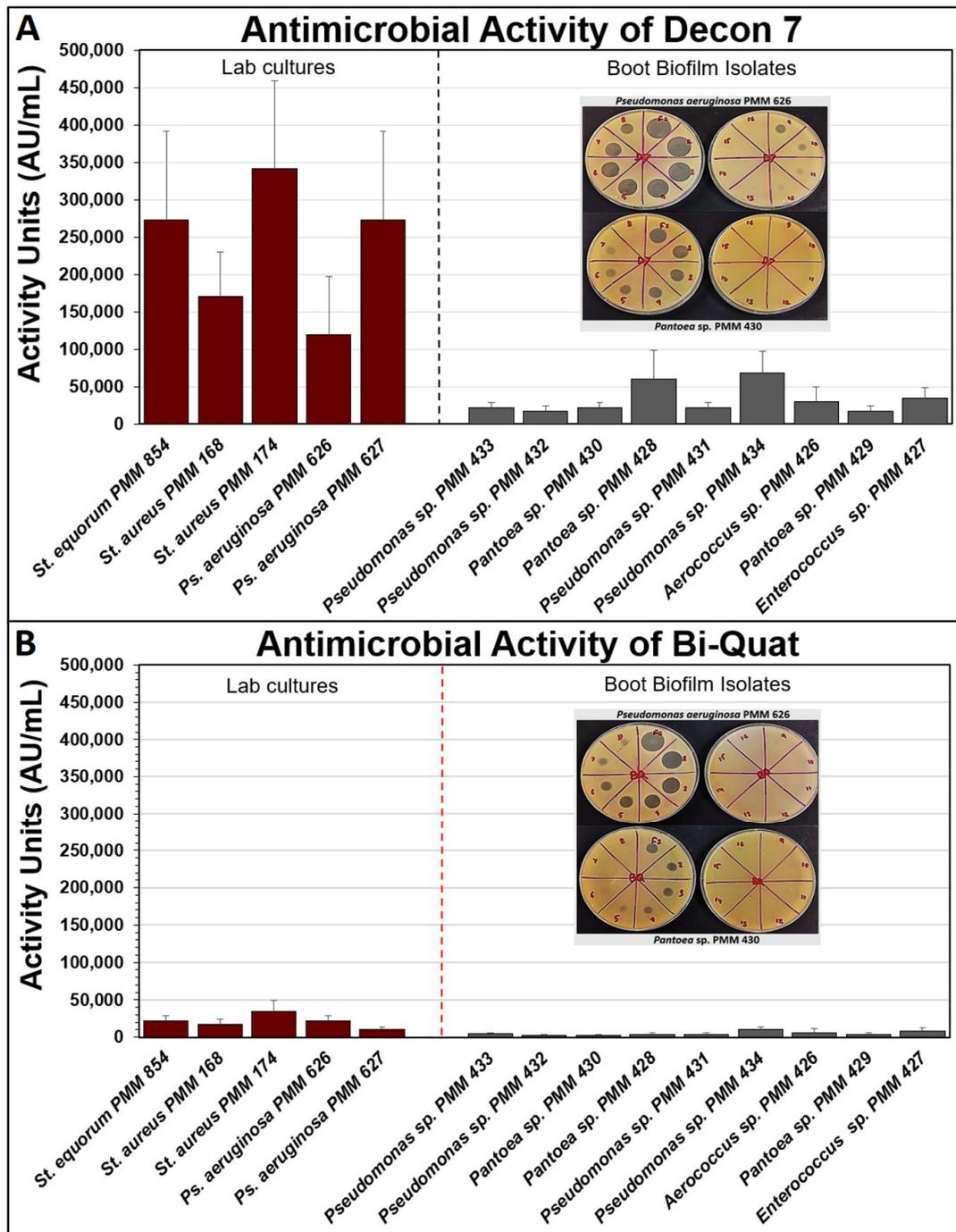


Figure 6. Antimicrobial susceptibility data from spot-on-lawn assays of Decon7 and Bi-Quat on laboratory biofilm cultures and bacteria isolated from workers’ boots from the FAPC abattoir. Data are presented as the means of triplicate replications, and error bars represent the standard deviation from the mean. Inset photos are typical representations of spot-on-lawn assays for Decon7 (A) and Bi-Quat (B) on *Ps. aeruginosa* PMM 626 and *Pantoea* sp. PMM 430.

4. Conclusions

Compared to other sanitizers that we have used, Decon7 is a next-generation sanitizer that possesses multiple modes of action, combining QACs, hydrogen peroxide, and a catalyst, providing the greatest log reduction in the shortest application time against *Listeria monocytogenes*, *E. coli* O157:H7, and *Salmonella* Montevideo [43]. In this study, it was also effective against laboratory biofilms of two strains of *Pseudomonas aeruginosa* that were problem isolates from commercial egg processing facilities, two strains of *Staphylococcus*

aureus, and one strain of *Staphylococcus equorum* associated with cow teats (Table 1). Decon7 also showed a reduction in the bacterial population when sprayed directly on workers' boots as compared to a first-generation sanitizer (Bi-Quat) that had been used daily for extended periods. The extended use of a sub-lethal application of this sanitizer for >20 years was a likely contributing factor that led to a buildup of alkaline-tolerant bacteria in biofilms on workers' boots. After comparing the sensitivity of *Pseudomonas* and *Staphylococcus* strains used in the microplate lethality assay vs. bacterial isolates recovered from the boot biofilms, the boot isolates were found to be more resistant to both Decon7 and Bi-Quat. The increased resistance to biocides and disinfectants is a concern in food industries and hence the development of new control strategies is strongly advocated. The repeated use of a weak sanitizer with a single chemistry (an early-generation QAC) imposed a selective pressure for surviving alkaliphilic bacteria. As bacterial biofilms develop, their extracellular (EPS) matrix of EPS increases sanitizer resistance not only through innate bacterial resistance, but also by producing an impenetrable layer that may protect entrapped bacteria. It is highly recommended that sanitizer chemistry be alternated to reduce the selection of antimicrobial resistivity in bacteria, which could lead to a buildup of biofilm bacteria where sanitizers are used. The periodic application of enzymes could also be carried out, as used in the recovery process for our microplates, to loosen and degrade bacterial extracellular matrices. It is only through the validation of antimicrobial treatments, as demonstrated in our boot study, that control regimens can be evaluated in terms of their efficacy.

Author Contributions: Conceptualization, P.M.M.; methodology, P.M.M. and K.S.; software, P.M.M.; validation, K.S.; formal analysis, K.S.; investigation, K.S.; resources, P.M.M.; data curation, P.M.M. and K.S.; writing—original draft preparation, K.S.; writing—review and editing, P.M.M.; visualization, P.M.M. and K.S.; supervision, P.M.M.; project administration, P.M.M.; funding acquisition, P.M.M. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by Decon7 (Scottsdale, AZ, USA), the Advance Foods/SE Gilliland Professorship in Microbial Food Safety (#21-57200), and the Oklahoma Agricultural Experiment Station Hatch Project (Project #OKL03090).

Institutional Review Board Statement: The study was conducted according to the protocol #16-5 (General Microbiology Lab Protocol) approved by the Oklahoma State University Institutional Biosafety Committee in accordance with the Centers for Disease Control and Prevention (USA), "Biosafety in Microbiological and Biomedical Laboratories", 6th Edition, USA.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data available upon request.

Acknowledgments: The authors acknowledge the Robert M. Kerr Food and Agricultural Products Center (FAPC) for in-kind support of labs and facilities. Thanks to Manish Aryal for isolating several bacterial from biofilm on workers' boots.

Conflicts of Interest: The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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