2019 Report

Application of Decon7 Against *Pseudomonas* and *Staphylococcus* Biofilms

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Objective.

Use recently improved microplate biofilm assay to demonstrate efficacy of Decon7 sanitizer against:

- *Pseudomonas* spp.
- *Staphylococcus* spp.

Methods.

1. Microplate biofilm assay for making biofilms with *Listeria,* STEC *E. coli*, and *Salmonella* has been published (attached):

    - Aryal, M.; Pranatharthiharan, P.; Muriana, P.M. Optimization of a Microplate Assay for Generating *Listeria monocytogenes*, *E. Coli* O157:H7, and *Salmonella* Biofilms and Enzymatic Recovery for Enumeration. *Foods* **2019**, *8*, 541.

2. Microplate biofilm assay using the 3 pathogens above employed using Decon7 is currently in press (attached):

    - Aryal, M.; Muriana, P.M. Efficacy of Commercial Sanitizers Used in Food Processing Facilities for Inactivation of *Listeria monocytogenes*, *E. coli* O157:H7, and *Salmonella* Biofilms. *Foods* **2019,** *8*, xxx.

3. The microplate biofilm assay described above was used to make biofilms with *Pseudomonas* spp. and *Staphylococcus* spp. and treated with Decon7.

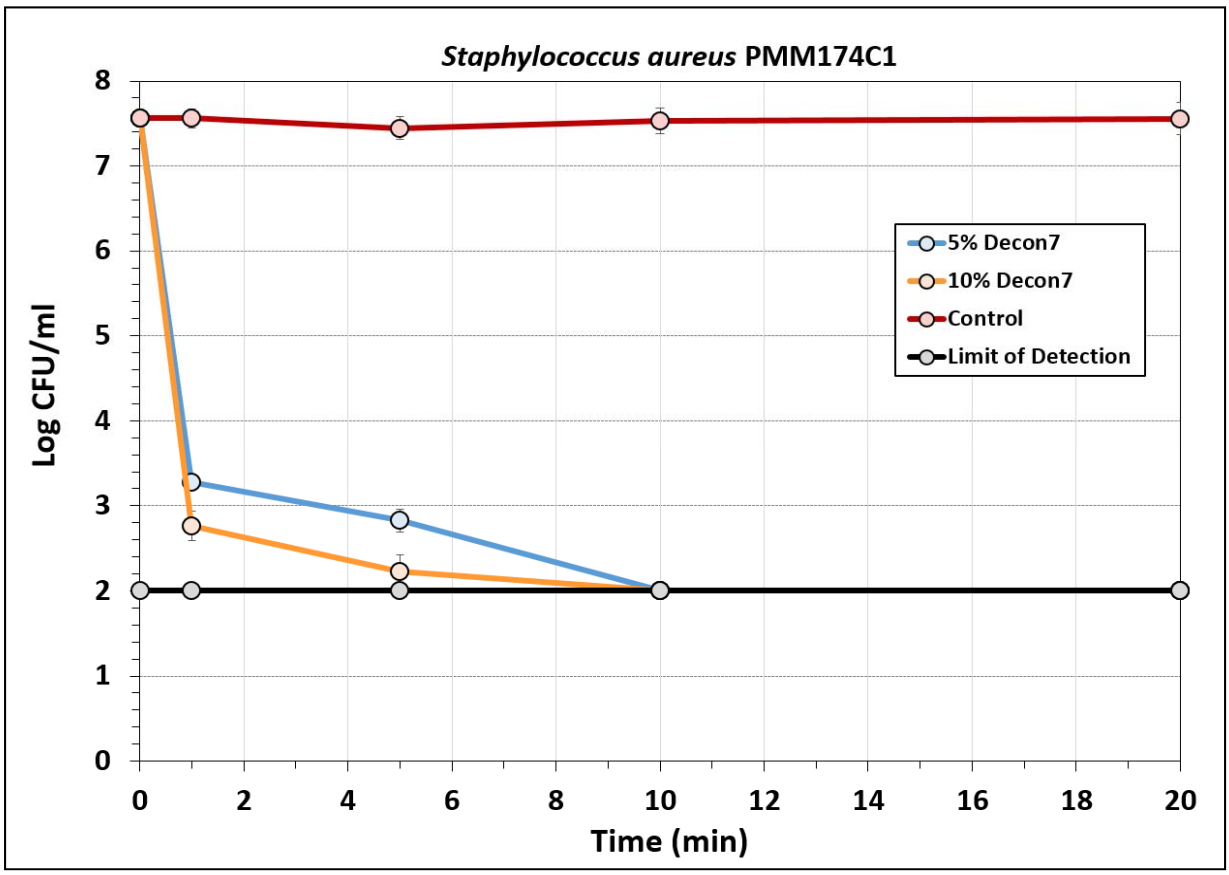
    Cultures. Cultures were from Dr. Muriana's Culture Collection, and included *Pseudomonas aeruginosa* 1, *Pseudomonas aeruginosa* 2, *Staphylococcus aureus* PMM 174C1, and *Staphylococcus aureus* PMM 169C8. Frozen cultures were thawed and transferred to 9 ml Tryptic Soy Broth (TSB), incubated overnight at 37°C, and sub-cultured twice before use. All assays were performed in triplicate replication; separate cultures were grown for each replication which were performed as autonomous experiments.

    Biofilms. Falcon 96-well microplates were used as the substrate/carrier to create biofilms. Overnight cultures (~10^9 cfu/ml) were diluted in TSB to ~10^5 cfu/ml and 200 uL of this culture dilution was added to microplates in triplicate replication. Different microplates were used for each Decon7 application time (i.e., 1- min, 5-min, 10-min, 20-min). Microplates were sealed with parafilm to avoid evaporation during growth of biofilms and incubated at 30°C for 24 hrs. After 24 hours, microplates were washed three times with sterile Tris buffer (pH 7.4, 0.05 M) in a Magna plate washer followed by the addition of fresh TSB (200 µL) into the wells containing the adhered bacterial strains. Plates were then sealed and incubated for 24 hours at 30°C. This process of washing and adding media into the microplates was continued daily for 7 days. After 7 days, microplates were washed three times with sterile Tris buffer. Treatments with Decon7 were then applied to the various biofilms (4 different strains, 2 strains of *Pseudomonas* and 2 strains of *Staphylococcus*). Both 5% [1 mL Decon7 mixture (2:2:1) in 20 mL sterile d/w] and 10% [1 mL Decon7 mixture in 10 mL sterile d/w] concentrations were prepared. 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 and then 200 µL of

Dey-Engley (D/E) neutralizing broth was then added and left for 5 min to neutralize any potential residual Decon7. After treatment for 5 min with D/E neutralizing broth, microplates were again washed with Tris buffer. 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 37°C for one hour, plated on Tryptic Soy Agar plates in duplicate, and incubated at 30°C for 48 hrs and enumerated. Microbial populations were converted to log denomination and plotted vs time (of treatment).

Results & Discussion.

This work was carried out based on our prior research. We used flat-bottom microplates (96-well) in combination with a plate washer and repeated addition of fresh media to remove planktonic/loose cells to create a 7-day enhanced biofilm of *Pseudomonas aeruginosa* 1 and 2, *Staphylococcus aureus* PMM 171C1, *Staphylococcus aureus* PMM 169C8, and *Staphylococcus equorum* PMM 854HS-7. 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 develops. This biofilm is irreversible and difficult to remove. It further disperses its cells to initiate the formation of a new biofilm. One of the major factor in biofilm formation of *Staphlococcus aureus* is said to be the expression of the icaA gene, which helps in the formation of a transcriptional product N-acetylglucosaminyl-transferase (Abdallah *et al.,* 2015). It 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 called "quorum sensing" in a biofilm to communicate among the bacterial cells. It helps in 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). Developing a fine 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 (BHI/TSA broth) each day for 7 days. The bacterial population in our biofilm achieved a 6.5-7.5-log CFU/ml level as determined by enzymatic detachment. Decon7 solution comes in three parts: A surfactant (quaternary ammonium compound), an oxidizer i.e. hydrogen peroxide and an accelerator (diacetin). After treating the 7-day old biofilm with Decon7, we observed approximately ~4-5 log reduction in *P. aeruginosa* 1 & 2, *S. aureus* PMM 174C1, *S. aureus* PMM 169C8, and *S. aureus* 854HS-7 within the first 1 minute of treatment (10% concentration).


Staphylococcus aureus PMM174C1
Log CFU/ml
Time (min)
5% Decon7
10% Decon7
Control
Limit of Detection
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3
4
5
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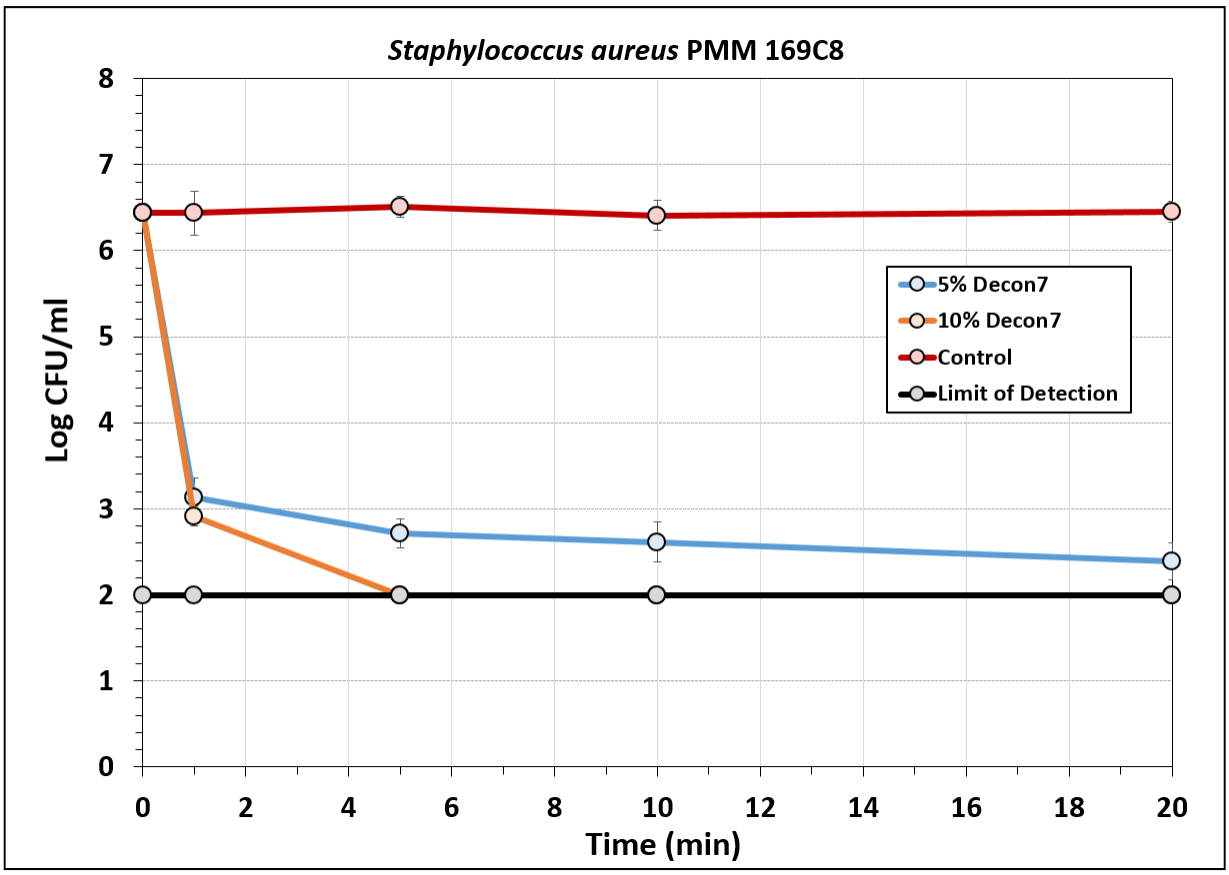
Staphylococcus aureus PMM 169C8
Log CFU/ml
Time (min)
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10% Decon7
Control
Limit of Detection
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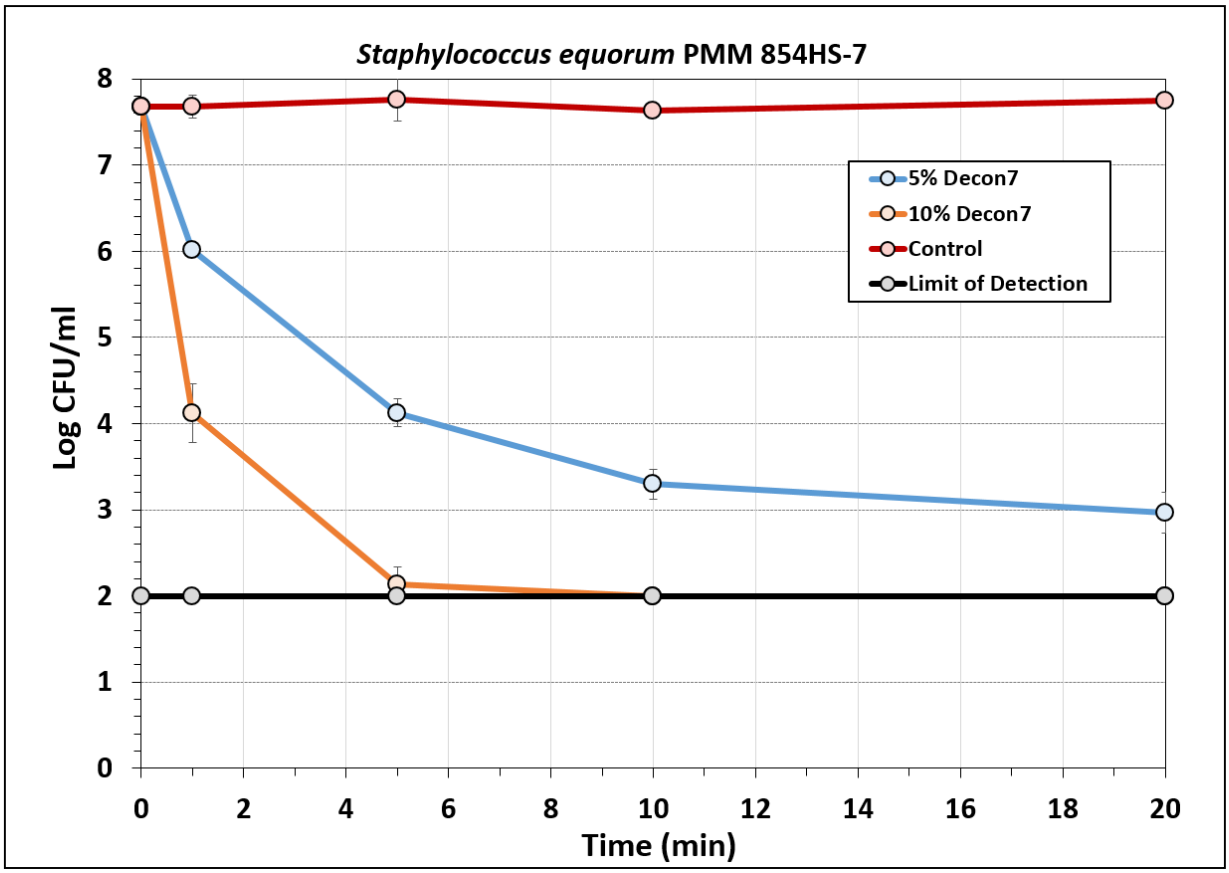
Staphylococcus equorum PMM 854HS-7
Log CFU/ml
Time (min)
5% Decon7
10% Decon7
Control
Limit of Detection
0
1
2
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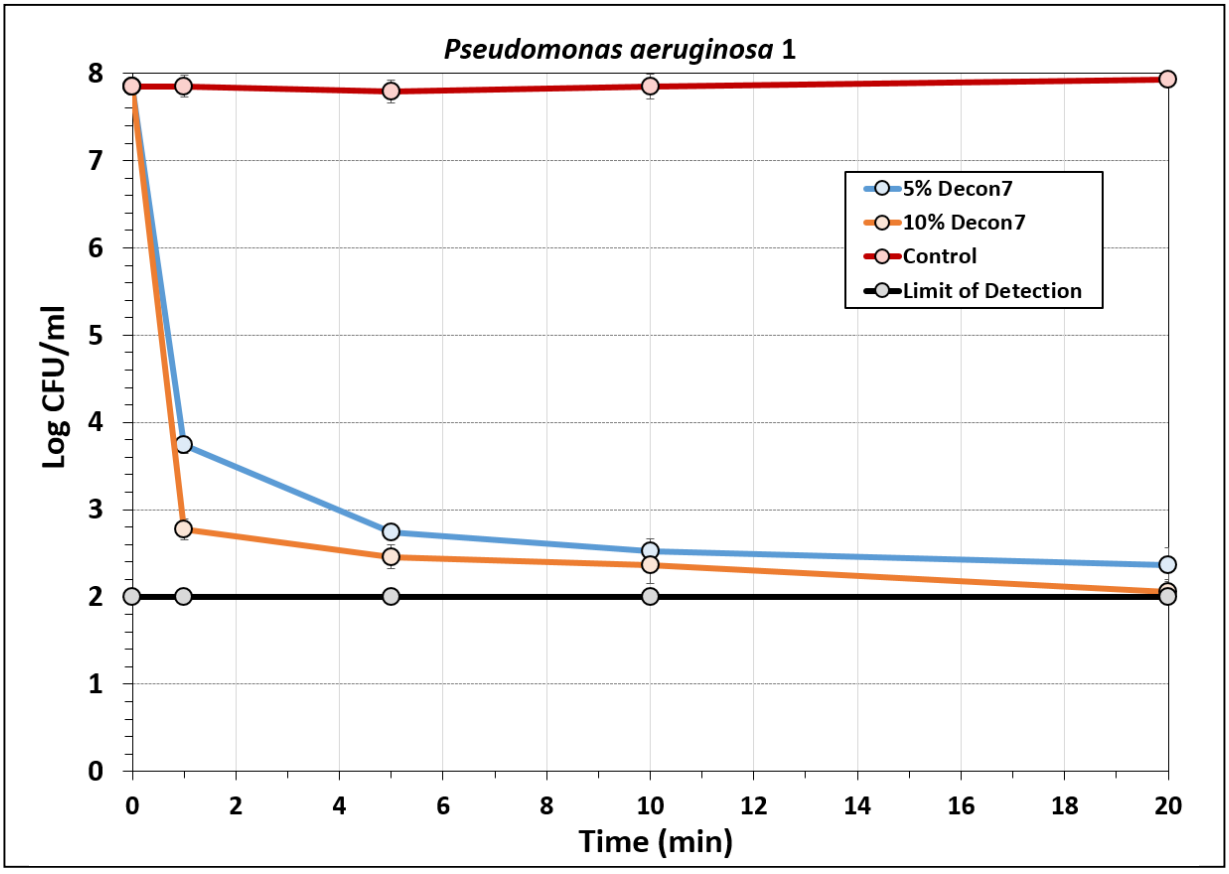
Pseudomonas aeruginosa 1
Log CFU/ml
Time (min)
5% Decon7
10% Decon7
Control
Limit of Detection
0
1
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3
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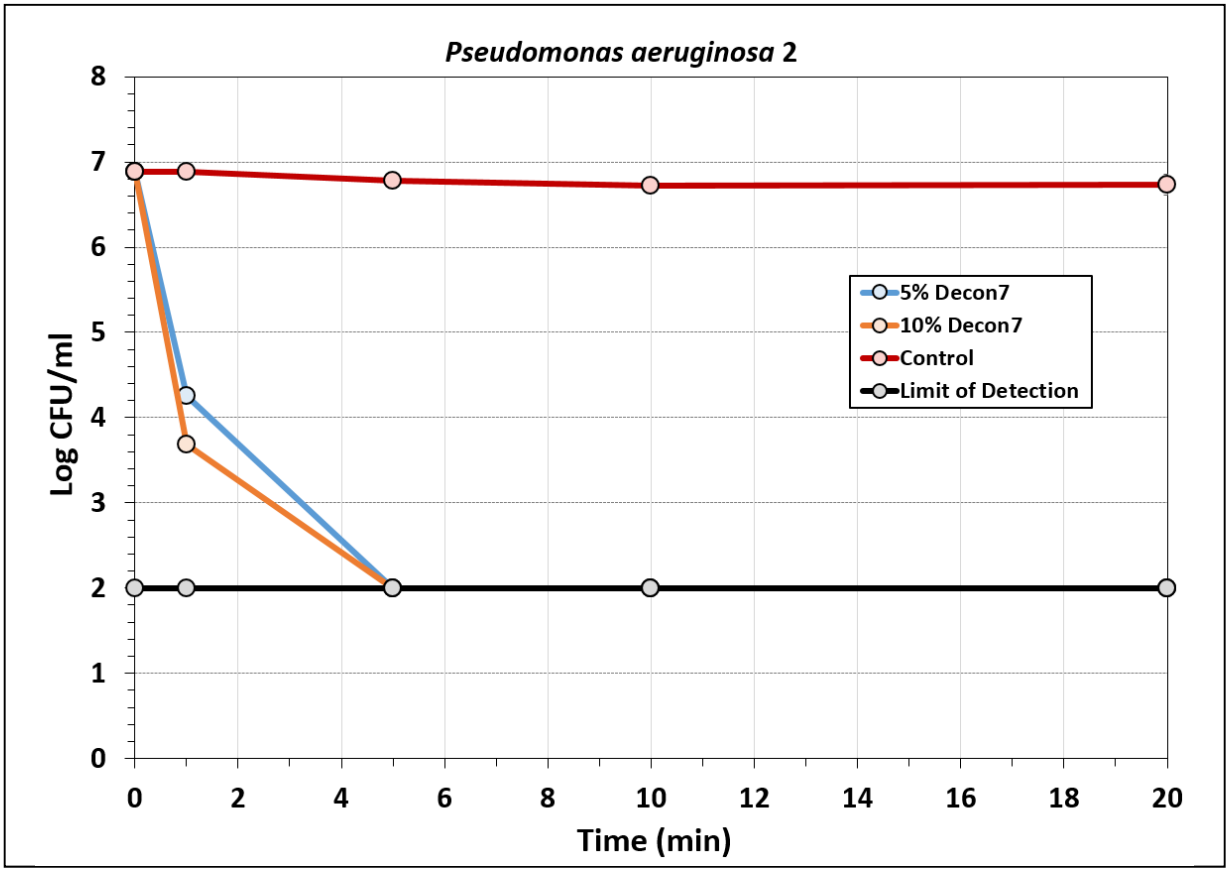

Conclusion. Compared to other sanitizers that we have used against *Listeria monocytogenes*, *E. coli* O157:H7, and *Salmonella* Montevideo, Decon7 provides the greatest reduction in the shortest time of application (Aryal and Muriana, 2019). We have further observed effective reduction of 2 strains of *Pseudomonas aeruginosa* that were problem isolates from egg pasteurization facilities as well as 2 strains of *Staphylococcus aureus,* and 1 strain of *Staphylococcus equorum*.

Further studies. Additional studies are being directed towards examination of practical application of Decon7 in our FAPC Meat Processing Pilot Plant. Currently, we are examining boots of student workers that have developed biofilms (generic) based on repeated-use of Bi-Quat (not highly effective; selects for alkaliphilic organisms).

Recommendation. We should have a discussion/conference call with Decon7 staff at the beginning of the new year to see if there is anything we can do to target research that is directly relevant to their needs.

foods

MDPI

Article

Efficacy of Commercial Sanitizers Used in Food Processing Facilities for Inactivation of *Listeria monocytogenes, E. coli* O157:H7, and *Salmonella* Biofilms

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Abstract: Bacteria entrapped in biofilms are a source of recurring problems in food processing environments. We recently developed a robust, 7-day biofilm microplate protocol for creating biofilms with strongly-adherent strains of *Listeria monocytogenes*, *Escherichia coli* O157:H7, and *Salmonella* serovars that could be used to examine the effectiveness of various commercial sanitizers. *Listeria monocytogenes* 99-38, *E.coli* O157:H7 F4546, and *Salmonella* Montevideo FSIS 051 were determined from prior studies to be good biofilm formers and could be recovered and enumerated from biofilms following treatment with trypsin. Extended biofilms were generated by cycles of growth and washing daily, for 7 days, to remove planktonic cells. We examined five different sanitizers (three used at two different concentrations) for efficacy against the three pathogenic biofilms. Quaternary ammonium chloride (QAC) and chlorine-based sanitizers were the least effective, showing partial inhibition of the various biofilms within 2 hrs (1-2 log reduction). The best performing sanitizer across all three pathogens was a combination of modified QAC, hydrogen peroxide, and diacetin which resulted in ~6-7-log reduction, reaching levels below our limit of detection (LOD) within 1-2.5 min. All treatments were performed in triplicate replication and analyzed by one way repeated measures analysis of variance (RM-ANOVA) to determine significant differences ($p<0.05$) in the response to sanitizer treatment over time. Analysis of 7-day biofilms by scanning electron microscopy (SEM) suggests the involvement of extracellular polysaccharides with *Salmonella* and *E. coli* which may make their biofilms more impervious to sanitizers than *L. monocytogenes*.

Keywords: Biofilm; sanitizer; *L. monocytogenes*; *Salmonella*; *E. coli* O157:H7; microplate assay

1. Introduction

Sanitary practices in food manufacturing facilities targets the elimination of pathogens and reduction of contaminating microbiota that gain access to the processing environment, often from the raw material and food ingredients, but also from workers, drains, and ventilation ducts [1-4]. Manufacturing shifts may process raw food ingredients for up to 8 hrs continuously. During this time, microorganisms may find harborages on food processing equipment and establish biofilms that may become the foci of contamination for manufactured foods or be displaced to other environmental locations. *Listeria monocytogenes, Bacillus cereus, E. coli* O157:H7, *Salmonella* spp., *Pseudomonas* spp., and *Staphylococcus aureus* have been documented to form biofilms on food and food contact surfaces [3,5,6]. Apart from a bacteria's ability to initiate attachment, intrinsic factors related to the chemistry of foods and extrinsic factors such as the food contact surface itself can influence the level of attachment and biofilm formation [7-9]. Bacteria may be influenced by different 'scale effects' of

surface topography (i.e., nano- vs micro-scale) that may affect attachment [10]. Early attachment may not be a completely random process, but rather may involve preferential attachment to sites that improve the chances of sustaining the development of biofilm [11]. These may involve various adhering chemistries of the external bacterial architecture such as hydrophilic/hydrophobic attractions from charged amino acids of flagella and pilli, or of carbohydrates and lipids[12-14]. However they may occur, biofilms in food manufacturing facilities can lead to contaminated foods resulting in outbreaks and illnesses among consumers [15].

Sinde and Carballo [16] found that the degree of attachment and efficacy of sanitizers on surface-biofilms varied considerably between surface types that may consist of various materials including stainless steel, glass, polyurethane, teflon, rubber, or wood [17,18]. In contrast, adherence characteristics could be strain-dependent as Gamble and Muriana [19] found strongly-adherent strains of *L. monocytogenes* that were equally-capable of forming biofilms on stainless steel, rubber, plastic, and glass while less-adherent strains were less capable of adhering to those surfaces. The attachment of pathogenic bacteria on these surfaces serves as a reservoir of microbial contamination and poses a high risk in the production line [20]. This has raised strong concerns for food safety and hence it is indispensable to develop proper cleaning and disinfection procedures for biofilm prevention and control [21].

In food industries, the disinfection of surfaces or equipment is mostly done by use of sanitizers [22,23]. There are different types of sanitizers which can be grouped broadly as oxidizing agents, surface active compounds, and iodophores [24]. Widely used sanitizers including halogen-based compounds, peracetic acid (PAA), ozone, and hydrogen peroxide fall within the group of oxidizing agents [23]. Surface active compounds such as acid anionic compounds and quaternary ammonium compounds (QACs) are also used abundantly in food industries [24]. However, the thick biofilm matrix comprised of fat, carbohydrates, nucleic acids and protein-based materials, limits the effectiveness of many sanitizers. Moreover, pH, temperature, contact time, water hardness, and concentration are also important factors influencing the effectiveness of disinfectants [25,26]. *Listeria monocytogenes* was shown to increase its resistance to QACs, chlorine and hydrogen peroxide when biofilm maturation time was increased [27]. Similarly, resistance of *E. coli* O26 clinical and cattle isolates to QACs and PAA-based sanitizers was observed when exposed for up to 2 min.[28] Some cells might have natural resistance and some might acquire resistance to the sanitizers through genetic exchanges or mutations [29]. Even more alarming are reports that suggest a correlation of biocide use with the development of antibiotic resistance [30,31]. Reviews by Kampf have shown that both Gram-negative and Gram-positive bacteria are equally capable of enhancing antibiotic resistance when subjected to biocidal use [32,33]. This is the reason that the US Food and Drug Administration banned the use of triclosan and other active agents from antimicrobial soaps for use at home by the general population. These capabilities possessed by some microbes allow them to grow and persist despite the application of sanitizers. Thus, increased resistance to biocides such as sanitizers, is a concern in food industries and hence the development of new control strategies is highly advocated [34].

Post-harvest processing by various industries (meat, dairy, vegetable) are known to have specific microbiota-associated biofilms. The dairy industry has bacteria associated with drains where nutritious spilled milk or whey results in a good growth environment in drains and problems have long been associated with tubing systems for pasteurization that have dead-end zones or difficult to clean by clean-in-place systems [35]. The vegetable and fruit juice industry often have acetic and citric acid acidified products which could lead to acid tolerant organisms contributing to biofilm formation [36]. Perhaps no food processing industry is as large and diverse as the meat industry, involving live animal operations, slaughter and beef carcass processing, to fabricated beef cuts, ground beef, and further processing as ready-to-eat (RTE) meats and all capable of being besieged by microbial biofilms [37].

We recently optimized a microplate method to facilitate the generation of extended biofilms produced by strongly adherent strains of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 and the subsequent recovery of remaining viable cells using enzymatic detachment [38]. The current work

describes our evaluation of five commercial sanitizers comprising the aforementioned types of sanitizers against biofilms of these three pathogens.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions.

E. coli O157:H7 F4546, *L. monocytogenes* 99-38, and *Salmonella* Montevideo FSIS 051 were previously screened by a microplate fluorescence adherence assay differentiating them from other strains by their high level adherence to form biofilms [19,38,39]. Active cultures were grown in Brain Heart Infusion broth (BHI, Difco, BD Laboratories, Franklin Lakes, NJ, USA) in 9 mL tubes at 30 °C. Cultures were harvested by centrifugation (6,000xg, 5 °C) of 9 mL of fresh, overnight cultures and cell pellets were resuspended in 2-3 mL of fresh sterile BHI broth containing 10% glycerol. Cell suspensions were placed into glass vials and stored in an ultra-low freezer (-80 °C). Frozen stocks were revived by transferring 100 µL of the thawed cell suspension into 9 mL of BHI broth, incubating overnight at 30 °C, and sub-cultured twice before use. Microbial enumeration for all assays was carried out on Tryptic Soy Agar (TSA, Bacto, BD), plated in duplicate.

2.2. Growth of Enhanced Biofilms in Microplates.

Overnight cultures (~9-log CFU/mL) of *E. coli* O157:H7 F4546, *L. monocytogenes* 99-38, and *S.* Montevideo FSIS 051 were diluted to ~4-log CFU/mL in BHI broth and 200 µL was added to sterile Falcon 96-well clear, non-treated, flat-bottomed polystyrene microplates (Cat# 351172, Corning, NY, USA) to initiate biofilms. Inoculated plates were incubated at 30 °C, washed daily with the microplate washer, and fresh sterile media added; this cycle of growth, wash, and media renewal was continued for 7 consecutive days in order to generate a robust 'extended' biofilm [38]. Microplate biofilms were washed in a plate washer to wash away planktonic cells and loosely-adhered cells in addition to re-suspending settled planktonic cells before further use (described below).

2.3. Washing Biofilms Generated in Microplates.

The microplates used for growing biofilms, and subsequently for sanitizer lethality assay and detachment of remaining viable cells, were subjected to a wash treatment in a Biotek Elx405 Magna plate washer (Ipswich, Suffolk, UK). This plate washer was connected to separate wash (10% disinfectant bleach solution, sterile de-ionized water, or sterile 0.05 M Tris buffer pH 7.4, depending on the need) and waste containers. The plate washer has 96 pairs of needles (a longer one for aspiration and a shorter one for dispensing) to draw liquids into, and out of, each of the wells and a shake parameter to shake the plate to re-suspend settled cells, or release loosely adhered cells, before washing. Before washing the 96-well microplates to which bacteria were adhered, maintenance cycles were performed to sanitize the plate washer needles and tubing by washing with 10% disinfectant bleach (2 times), followed by de-ionized water (3 times), and Tris buffer (2 times). After the rinses, microplates with adhered cells were washed with 0.05 M Tris buffer (pH 7.4) for 3 times, each time using the shake option in the Elx405 plate washer.

2.4. Enzymatic Detachment of Adhered Cells from Microplates for Enumeration.

A trypsin enzyme solution (Cat: T4549; 1486 U/mL; Sigma-Aldrich, St. Louis, MO, USA) of 500 U/mL from porcine pancreas was used to release adhered *L. monocytogenes*, *E. coli*, and *Salmonella* in order to obtain a plate count enumeration of biofilm-adhered bacteria, either before (controls) or after sanitizer treatment (experimental). After the final wash with 0.05 M Tris buffer (pH 7.4), 200 µL of trypsin enzyme solution was transferred into the experimental wells. After the addition of enzyme, the microplate was incubated for 1 hr at 37 °C. Finally, to get detached cell counts, the solutions from the wells were surface plated on TSA plates and incubated at 30 °C for 24-36 hours. The limit of detection (LOD) of plate counts from microplate wells was observed at 2-log CFU/mL (1:10 dilution of trypsinized cells from microplate wells followed by surface plating of 0.1 mL).

2.5. Sanitizers Used in the Microplate Biofilm Assay.

Different common and new generation sanitizers (Bi-Quat, 10-Chlor, Sterilex, KC-610, and Decon7) were used in this study to analyze their effects on biofilms (Table 1).

Bi-Quat (Birko, Henderson, CO, USA) was used at a concentration of 200 ppm (i.e. 0.08 gal per 40 gal of water, or 2 mL per 1 liter of water) and 1,000 ppm. The effects of Bi-Quat on pathogenic biofilms were observed over the time periods of 0-, 15-, 30-, 60-, and 120 min.

Chlorine-based 10-Chlor (Birko; 10% sodium hypochlorite) was used in two different concentrations of 200 ppm (2.5 oz. per 10 gal of water) and 1000 ppm (12.5 oz. per 10 gal of water). The biofilms were separately incubated with 200 ppm and 1000 ppm of 10-Chlor for five different time periods; 0-, 5-, 15-, 30-, and 60 min.

Sterilex solution (Sterilex Corporation, Cockeysville, MD, USA) is a two part liquid concentrate mixed together at the time of use. The two different parts are: Part 1 (Ultra Disinfectant Cleaner Solution 1) and Part 2 (Ultra Activator Solution). We used two different concentrations of working Sterilex sanitizer solution, 5% and 10%. The biofilm treatment time periods were 0-, 1-, 2.5-, 5-, 10-, and 20 min for the 10% solution, and 0-, 2.5-, 5-, 10-, and 20 min for 10% solution.

Decon7 solution (Decon™ Seven Systems, Scottsdale, AZ, USA) came in three parts: Part 1: A surfactant (quaternary ammonium compound); Part 2: An oxidizer (hydrogen peroxide); and Part 3: An accelerator (diacetin). These three parts were mixed in the ratio 2:2:1 to form the stock solution. Working solutions were made at 5% and 10% concentration of the stock solution to assess efficacy against biofilms. Similar to Sterilex solutions, the 5% Decon7 solution had treatment time periods of 0-, 2.5-, 5-, 10-, and 20 min, while the 10% Decon7 solution was used with treatment times of 0-, 1-, 2.5-, 5-, 10-, and 20 min.

KC-610 (Packers Chemical, Kieler, WI, USA) is a peroxyacetic acid (PAA)-based antimicrobial solution which was used as per the manufacturer's instructions at a concentration of 6.1 oz. per 6.0 gal of water. The active ingredients of the solution were 5.6% peroxyacetic acid and 26.5% H_2O_2. The treatment time periods for this chemical were assigned at 0-, 5-, 15-, 30-, and 60 min.

Table 1. Sanitizers used in this study.

Trade Name	Active Ingredients	Use Level	Source
Bi-Quat	Dimethyl ethylbenzyl ammonium chloride (5.1%); Alkyl dimethyl benzyl ammonium chloride (5.1%); Ethanol (1.1%)	200 ppm, 1000 ppm	Birko Corp.
10-Chlor	Sodium hypochlorite (<20%); Sodium hydroxide (<5%)	200 ppm, 1000 ppm	Birko Corp.
Sterilex solution	1.Ultra Disinfectant Cleaner: Hydrogen peroxide (5.5-7.2%), Alykl dimethyl ethyl benzyl ammonium chloride (2.5-3.5%), Alkyl (C12,C14,C16) dimethyl benzyl ammonium chloride (2.5-3.5%) 2. Ultra Activator Solution: Sodium carbonate (4-8%); Potassium carbonate (4-8%); Tetrasodium ethylenediaminetetraacetate (3-7%)	5%, 10%	Sterilex Corp.
KC-610	Peroxyacetic acid (5-6%), Hydrogen peroxide (25-58%), Acetic acid (5-10%)	500 ppm	Packers Chemical
Decon7 solution	1.Quaternary ammonium chloride Benzyl-C12-C16 Alkyl Di-methyl Chlorides (5.5-6.5%); 2. Hydrogen peroxide (<8%); 3. Accelerant: Diacetin (30-60%)	5%, 10%	Decon7 Systems

2.6. Microplate Biofilm Sanitizer Assay.

Biofilm lethality assays using various sanitizers were carried out in 96-well microplates. *Listeria monocytogenes* 99-38, *E. coli* O157:H7 F4546, and the *S.* Montevideo FSIS 051 were used to form 7-day old mature biofilms (microplates were washed daily, sterile media replaced so only the adhered cells contribute to further growth). The 7-day biofilms were washed 3x with Tris buffer (0.05M, pH 7.4) in the plate washer (with shaking) and 250 μL of different concentrations of various sanitizers were added (or Tris buffer for controls). After the sanitizer (or buffer) incubation periods, the microplates

were again washed with Tris buffer, aspirated, and then 250 μL Dey-Engley (DE) neutralizing buffer (Hardy Diagnostics, Santa Maria, CA) was added to the wells and left for 5 min to neutralize the effects of sanitizers. The microplates were then washed with Tris buffer (0.05 M. pH 7.4) in a the plate washer and 250 μL of trypsin (500 U/mL) was added into the wells and incubated for an hour at 37 °C. The solution from the trypsin-treated biofilm-containing wells was harvested and plated on TSA plates. The plates were then incubated for 24-36 hours at 30 °C and enumerated the next day (24-30 hrs).

2.7. Scanning Electron Microscopy (SEM) of Biofilms.

Biofilms of the 3 pathogens used in this study were examined by scanning electron microscopy (SEM) by inoculating ~4-log CFU/mL in BHI broth (250 μL) into wells of Millicell EZ Slide 8-well glass slides (Millipore Sigma, Sheboygan Falls, WI, USA), sealed with parafilm to avoid evaporation, and incubated at 30 °C; the media (BHI) and planktonic cells in the wells was manually removed, washed, and replenished each day as described earlier for microplates, in order to achieve 7-day extended biofilms in the wells. A standard protocol provided by Oklahoma State University's Electron Microscopy lab was used to fix, dry, and coat the samples before imaging. Cells were fixed for 2 h in 2.0% glutaraldehyde in 0.1 M cacodylate buffer (21.4 g sodium cacodylate brought to 500 mL with deionized H_2O). The slides were then rinsed 3x in buffered wash (60 mL of 0.2 M cacodylate buffer, 140 mL of dH_2O, and 12.3 g of sucrose; 15 min/rinse). Adherent cells were again fixed for 1 h in 1% aqueous osmium tetroxide (OsO_4) at room temperature and then rinsed 3x in buffered wash solution (15 min/rinse). This was followed by dehydration in ethanol of different concentrations: 50%, 70%, 90%, 95%, and 100% (3x, 15 min/step) and then the slide(s) were subjected to critical point drying (CPD) or washed 2x for 5 min with HMDS (Hexamethyldisilazane). Silver paint or double-sticky tape was used to mount on stubs which were then coated with gold-palladium (Au-Pd) and either visualized, or stored in a dust-free dry area (desiccator) to view later. Visualization of the biofilms was done using an FEI Quanta 600 FEG scanning electron microscope (SEM) at the Oklahoma State University Electron Microscopy Core Facility.

2.8. Statistical Analysis.

Each trial was performed in triplicate replication and all replications were performed as autonomous and separate experiments using separately inoculated cultures and prepared plating media. All data were presented as the mean of triplicate replications and standard deviation of the mean is represented by error bars. Statistical analysis of timed-series plots was done by repeated measures one way analysis of variance (RM-ANOVA) using the Holm-Sidak test for pairwise multiple comparisons to determine significant differences of sanitizer treatment on biofilms over time. Data treatments with different letters are significantly different ($p < 0.05$); treatments with the same letter are not significantly different ($p > 0.05$).

3. Results

3.1. Sanitizer Biofilm Microplate Assays vs L. monocytogenes, Salmonella Montevideo, and E. coli O157:H7.

3.1.1. Hypochlorite-Based Sanitizer.

A hypochlorite-based sanitizer (10-Chlor) was used against 7-day biofilms of *E. coli* F4546, *S.* Montevideo FSIS 051, and *L. monocytogenes* 99-38 at 2 different concentrations (200- and 1000 ppm) with plate counts representing trypsin-recovered cells after 0-, 5-, 15-, 30-, and 60-min treatment time (Figure 1). The use of 200 ppm 10-Chlor resulted in a minimum reduction of all three pathogens even when used for as long as 60-sec of immersion (Figure 1A). However, when levels were increased to 1000 ppm, *L. monocytogenes* dropped to undetectable levels between 5- and 15-min, *E. coli* F4546 slowly dropped to undetectable levels by 60-min, while *Salmonella* was not affected much more than it was at 200 ppm (Figure 1B).

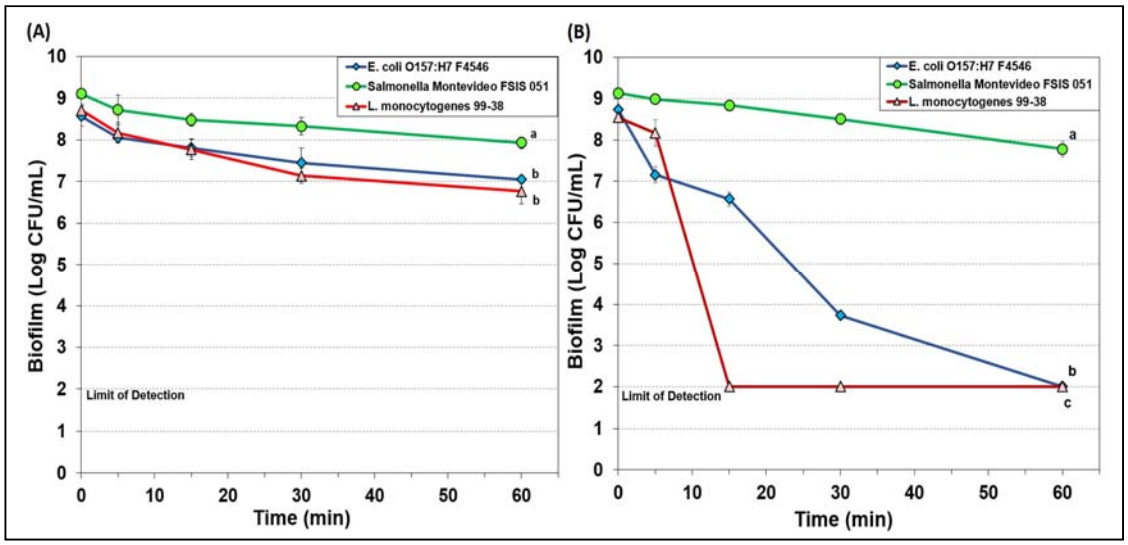

Figure 1. Biofilm microplate assay of *E. coli F4546, S.*Montevideo *FSIS 051, and L. monocytogenes* 99-38 against 7-day biofilms challenged with 10-Chlor sanitizer for 0-60 min at either 200 ppm (**A**) or 1000 ppm (**B**). Data points represent the means of triplicate replications and error bars represent 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, $p > 0.05$).

3.1.2. Simple Quaternary Ammonium Chloride-Based Sanitizer.

A simple QAC sanitizer (Bi-Quat) was used at 200- (Figure 2A) and 1000-ppm (Figure 2B) on biofilms of the three pathogens for 0-, 15-, 30-, 60-, and 120-min. Biofilms of *L. monocytogenes* 99-38 was most sensitive to Bi-Quat at both concentrations, and readily demonstrated nearly a 5-log decrease with 200-ppm within 15 min (Figure 2A); at 1,000-ppm, a >7-log decrease was observed with the same treatment time (Figure 2B). However, similar to the situation with 10-Chlor, *E. coli* O157:H7 F4546 and *S.* Montevideo FSIS 150 were more resistant to Bi-Quat showing barely a 1- and 2-log reduction, respectively, with 200-ppm after 2 hr treatment time (Figure 2A). Although 1,000-ppm was effective in providing greater reductions of *Salmonella* and *E. coli* O157:H7, it did not completely inactivate them in biofilms (Figure 2B).

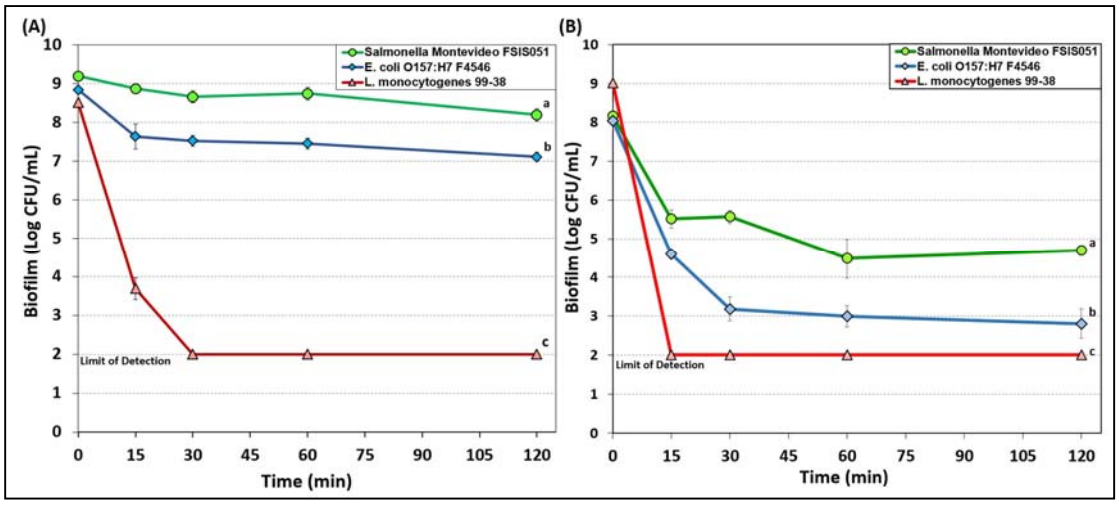

Figure 2. Biofilm microplate lethality assay of *E. coli F4546, S.* Montevideo *FSIS 051, and L. monocytogenes* 99-38 on 7-day extended biofilms challenged with Bi-Quat sanitizer for 0-120 min at 200-ppm (**A**) and 1000-ppm (**B**). Data points represent the means of triplicate replications and error 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, $p > 0.05$).

3.1.3. Peroxyacetic acid-based sanitizer.

Peroxyacetic acids are becoming more popular for use as sanitizers as microbial problems persist with recurring environmental contamination. We examined KC-610 sanitizer (at 500-ppm) against enhanced biofilms of all three pathogens for durations as long as 60 min (Figure 3). Biofilms of both *L. monocytogenes* 99-38 and *E. coli* O157:H7 F4546 were quickly reduced to below detectable levels (>7.5-log reduction) within 5 min of application while *S.* Montevideo FSIS 051 was reduced less than 3 logs within 5 min but to undetectable levels by 30-min (Figure 3).

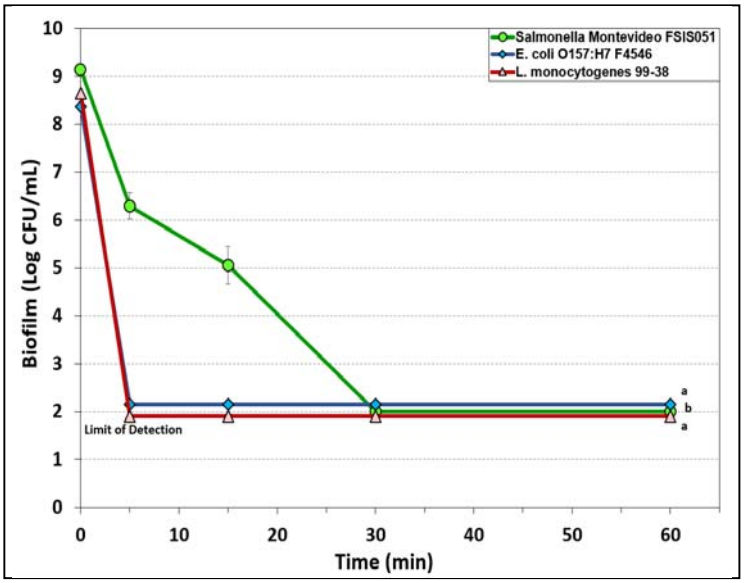

Figure 3. Biofilm microplate lethality assay of extended biofilms of *E. coli F4546, S.* Montevideo FSIS 051*, and L. monocytogenes* 99-38 on 7-day biofilms challenged with 500 ppm KC-610 PAA sanitizer for up to 60 min. Data points represent the means of triplicate replications and error bars represent 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, $p > 0.05$).

3.1.3. New Generation Quaternary Ammonium Chloride-Based Sanitizers.

A 'new generation' sanitizer, Sterilex Ultra, consisting of a 2-part sanitizer including a hydrogen peroxide/QAC solution and an 'activator' solution which, after formulation, was used at 5% and 10% strength. Both concentrations acted quickly and rapidly on biofilms of *L. monocytogenes* 99-38, with 5% and 10% formulations reducing *L. monocytogenes* to undetectable levels (>6-log reduction) in 2.5- and 1-min, respectively (Figure 4). Biofilms of *E. coli* O157:H7 F4546 were not affected as much as those of *L. monocytogenes* 99-38, and application of a 5% solution showed a < 3-log reduction in 10 min that remained approximately the same through 20 min (Figure 4A) while a 10% formulation slowly decreased *E. coli* O157:H7 F4546 to > 6.3-log reduction in 20 min (Figure 4B). *Salmonella* biofilms remained more resistant to Sterilex Ultra, observing only a 1.7-log decrease with 5% formulation (Figure 4A) and a 2.1-log reduction with 10% solution through 20-min (Figure 4B).

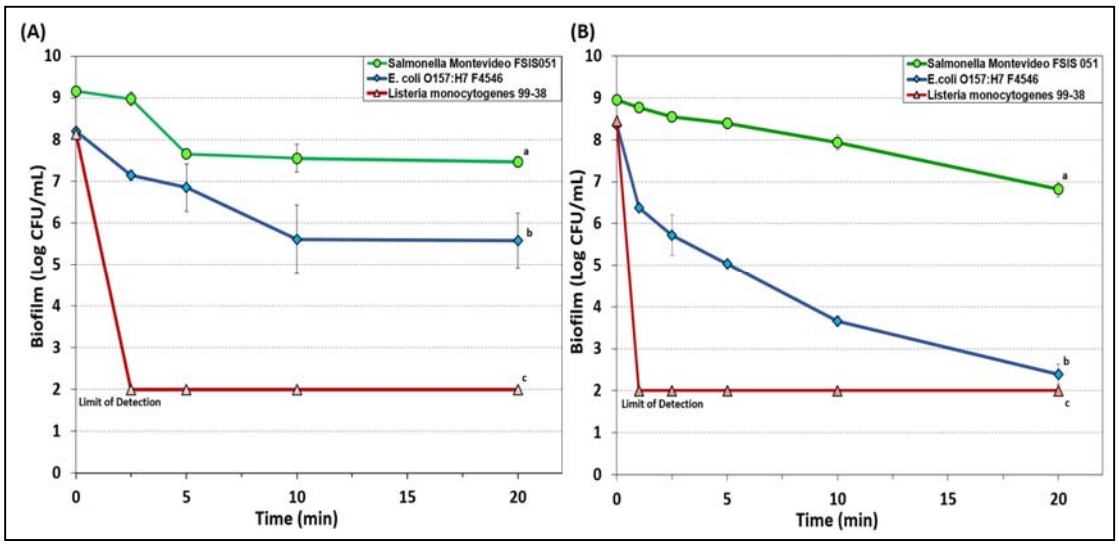

Figure 4. Biofilm microplate lethality assay of extended biofilms of *E. coli* F4546, *S.* Montevideo FSIS 051, *and L. monocytogenes* 99-38 against 7-day biofilms challenged with 5% (A) and 10% (B) solutions of Sterilex Ultra sanitizer for up to 20 min. Data points represent the means of triplicate 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, $p > 0.05$).

Another new generation QAC-based sanitizer was Decon7 that is a 3-part solution formulation consisting of a surfactant (quaternary ammonium compound), an oxidizer (hydrogen peroxide) and an accelerator (diacetin). Decon7 was also used at both 5% and 10% concentrations on biofilms of each of our three pathogens. At 5% solution, Decon7 worked quickly to reduce both *L. monocytogenes* and *E. coli* O157:H7 to undetectable levels (i.e., >6-logs) while *Salmonella* incurred a 3-log reduction and persisted even after 20 min (Figure 5A). When applied at 10% concentration, all three pathogens were reduced to below detectable levels with even the resistant *Salmonella* reaching ~7-log reduction within 2.5 min of treatment (Figure 5B).

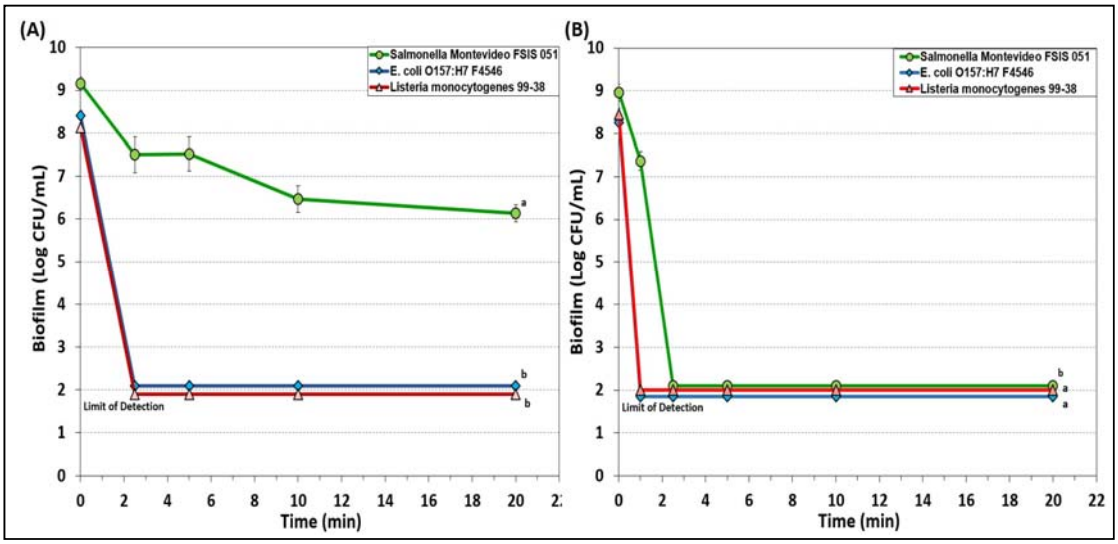

Figure 5. Biofilm microplate lethality assay of extended biofilms of *E. coli* F4546, *S.* Montevideo FSIS 051, *and L. monocytogenes* 99-38 7-day challenged with 5% (A) and 10% (B) solutions of Decon7 sanitizer for up to 20 min. Data points represent the means of triplicate replications and error bars 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, $p > 0.05$).

3.2 Scanning Electron Microscopy of Biofilms of L. monocytogenes 99-38, E. coli O157:H7 F4546, and S. Montevideo FSIS 051.

The 7-day biofilms for *L. monocytogenes* 99-38, *E. coli* F4546, and *S.* Montevideo FSIS 051 were also visibly different when examined by SEM (Figure 6). The *Listeria* looked like clean, smooth bacterial cells (Figure 8A) while the *E. coli* (Figure 8B) and *Salmonella* (Figure 8C) appeared to be coated with a film. Each of these biofilms provided >8 log CFU/mL in 200-300 ul when recovered from microplate wells (with trypsin) and enumerated on petri plates.

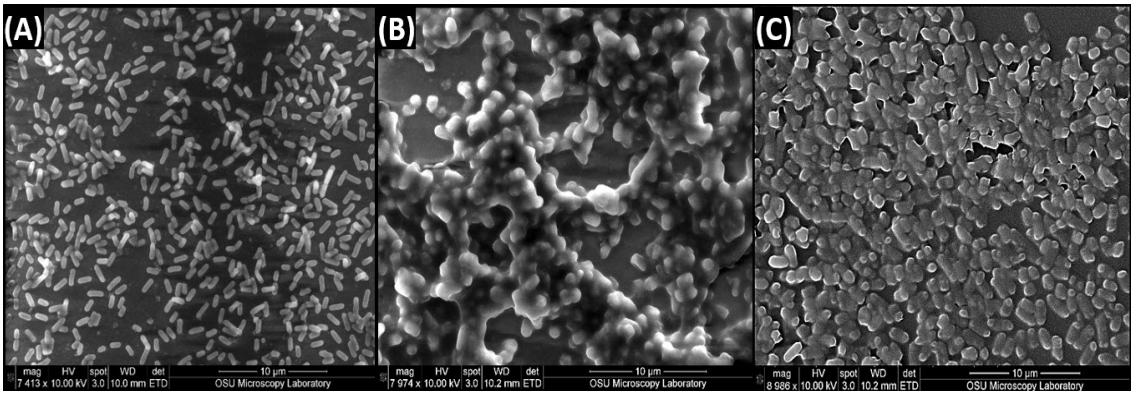

Figure 6. Scanning electron microscopy (SEM) of enhanced 7-day biofilms prepared on slide chambers from (A) *Listeria monocytogenes* 99-38, (B) *E. coli* O157:H7 F4546, and (C) *S.* Montevideo FSIS 051. Approximately 7,000-9,000-fold magnification.

4. Discussion

During our prior work on developing robust, 7-day enhanced biofilms with three strongly-adherent pathogens, we optimized conditions for biofilm formation (7-consecutive days of washing/renewing growth media), fluorescence substrate (5,6-CFDA selected as the better substrate), and enzymatic recovery (trypsin enzyme) from adhered biofilms [38]. Additional studies had indicated to us that a common sanitizer (Bi-Quat) used in our in-house slaughter facility was not effective on *E. coli* O157:H7 biofilms nor effective against pre-existing biofilms that had developed on workers boots. This provided the impetus to examine the effectiveness of five commercial sanitizers against robust biofilms on our microplate biofilm platform under standardized conditions of use.

Chlorine-based solutions are the most common and inexpensive sanitizers used in food industries and hence the efficacy of other sanitizers is often evaluated by comparison with chlorine-based sanitizers [40]. In the USA, chlorine is used for sanitization of equipment as well as poultry meat even though reactive chlorine has been shown to generate potentially carcinogenic chlorinated byproducts (trihalomethane, semicarbazide) [41,42]. Because of this, the use of chlorine has been drastically curtailed in other parts of the world, particularly in the European Union, and even in the USA there are strict limits on levels of free chlorine in industrial waste streams.

Commonly used chlorine sanitizing compounds include liquid chlorine, hypochlorites, and chloramines. Chlorines are strong oxidizing agents and broad spectrum germicides which have a variety of modes of action of disinfection. They are found to act on microbial membranes, oxidize sulfhydryl enzymes, hinder DNA synthesis and damage DNA, oxidize respiratory components, inhibit protein synthesis and act by a combination of factors acting simultaneously [43]. However, as oxidizing compounds, they are readily rendered inactive depending on the availability of organic reducing material [44].

The application of 10-Chlor (hypochlorite) at low concentration was largely ineffective and the biofilm organic layer may have reduced the low level of oxidizer (hypochlorite) present as 200 ppm (Figure 1A). Increasing the concentration to 1,000 ppm overcame the inactivation of the active agent yet still showed differences possibly based on sensitivity/resistance of the target organisms (Figure 1B).

Quaternary ammonium compounds (QACs) are cationic surface active agents (surfactants) that contain a centrally placed nitrogen atom covalently bonded with four alkyl (R) groups and a negatively charged anion portion [45]. The activity of QACs is the result of cationic charges that form electrostatic bonds with negatively charged bacterial proteins [46] and application of such antimicrobials involves interaction with membrane proteins, disruption of membrane integrity and leakage of cytoplasmic contents [47]. QACs are stable, active, possess low toxicity and have higher efficacy against Gram-positive bacteria, yeasts, molds, and lipid-containing viruses. They are however not as effective against Gram-negative bacteria, endospores, and bacteriophages [45,48]. The nature and length of alkyl (R) groups determine the antimicrobial activity of QACs with methyl group of 12 to 14 carbon chain showing greater activity [45]. In the USA, CFR Title 21 restricts use of quaternary ammonia compounds to 200 ppm on food contact surfaces. Bi-Quat is an example of early generation QAC sanitizers that have been widely used in the food industry. In our studies, 200-ppm Bi-Quat was effective against *L. monocytogenes*, but showed limited effectiveness against *E. coli* and *Salmonella* unless used at 1,000 ppm (Figure 2).

Peroxyacetic acid (PAA) is also simply known as peracetic acid and is a stronger oxidizing agent than chlorine. Commercially available PAA is the equilibrium form of a quaternary mixture of acetic acid, hydrogen peroxide, PAA, and water [49]. The popularity of PAA as a sanitizer is due to its scope of action against bacteria, yeast, and fungi, its decomposition into harmless byproducts, and it's application over a wide range of temperature (0-40 °C) and pH (3-7.5) [48]. Federal regulations prohibits the use of PAA above 200 ppm for food contact surfaces although higher levels may be used if subsequently rinsed with water. The mode of action of PAA like any other oxidizing agent is denaturing proteins, dislocating or rupturing the cell wall, and oxidizing sulfhydryls and sulfur bonds in enzymes and other metabolites [50]. Peracetic acid has been found to eliminate viable *S. aureus* (reduction by 98%) and *P. aeruginosa* (99% reduction) on surfaces with only 1 min of contact time but was not effective against the same bacteria in biofilms [51]. In our study, PAA (KC-610) was very effective in reducing *L. monocytogenes* and *E. coli* O157:H7 to undetectable levels (i.e., > 6.5-log reduction) and ~3-log reduction of *Salmonella* within 5 min (Figure 3).

Hydrogen peroxide is a clear, colorless liquid and an environment friendly (non-toxic) sanitizer widely used in the medical field and in food industries. It is effective against a broad spectrum of microorganisms including viruses, bacteria, bacterial endospores and yeasts [15,50] and the primary mode of action is through oxidization and production of hydroxyl (•OH) free radicals. These free radicals can attack and disrupt membrane lipids, target DNA and proteins (sulfhydryl bonds) and affect other essential cellular components [50]. Hydrogen peroxide is extensively used in produce industries to sanitize surfaces of whole and fresh cut melons [52]. Activity is further enhanced in combination with new generation QAC's including products like Sterilex and Decon7 that combine the effectiveness of multi-quaternary ammonium compounds with hydrogen peroxide. Our data on extended biofilms showed that Sterilex was very effective against *L. monocytogenes* 99-38 (> 6-log reduction in 2.5 min at 5% strength), moderately effective against biofilm of *E. coli* O157:H7 F4546 (< 3-log reduction in 2.5 min at 10% strength), and least effective against *S.* Montevideo FSIS 051 (~1-log reduction in 10 min at 10% strength)(Figure 4). Decon7 is similar to Sterilex, but it also includes diacetin (glycerin diacetate) as an 'accelerator' which appears to provide additional effectiveness against biofilms as evidenced by our data, achieving > 6-log reduction of *L. monocytogenes* and *E. coli* O157:H7 within 1 min (10% strength) and > 7.5-log reduction of *Salmonella* in 2.5 min (Figure 5).

Examination of the data for the various sanitizers on the biofilms used in this study demonstrates differences between the effect on *L. monocytogenes* 99-38 (most sensitive) vs *E. coli* F4546 and *S.* Montevideo FSIS 051 (less sensitive) (Figures 1-5). When biofilms were examined by SEM, we observed that *L. monocytogenes* 99-38, despite having been enriched by seven consecutive days of

washing/growth, were observed as 'clean' cells fixed to the surface (Figure 6). However, those of *E. coli* O157:H7 F4546 and *S.* Montevideo FSIS 051 appeared as if covered with a coating. The results showing less sensitivity to the sanitizers suggests that protection might be afforded by the EPS produced by these organisms (Figure 6). Such structures have been observed by others and not only acts as a 'glue' that holds the biofilm together, but also as a protective coating that restricts diffusion of nutrients and/or antimicrobials from reaching retained and embedded cells [53,54].

5. Conclusions

The array of published data concerning the inactivation of microorganisms with sanitizing disinfectant antimicrobials is lengthy and overwhelming [55-57]. Microbial susceptibility to sanitizers can largely depend on whether the cells are loosely available or if they are buried within the intricacies of a biofilm. In order to develop a standardized robust biofilm for testing purposes, we screened for the most strongly-adherent stains of 3 different pathogens, and applied them in an extended microplate biofilm assay by daily removal of planktonic cells and re-application of fresh media daily for 7-days [38]. During our application of commercial sanitizers within this standardized platform, we have observed that some are more effective than others and over short or longer application times (Figures 1-5). In all cases, it appears that *L. monocytogenes* 99-38, even when presented as a biofilm, is the most sensitive of the 3 pathogens we have tested while *E. coli* F4546 and *S.* Montevideo FSIS 051 are much less sensitive. This could very well be due to the fact that *L. monocytogenes* does not make EPS (appears as smooth naked cells, Figure 6) while both *E. coli* and *Salmonella* known to make EPS and are observed as covered with a coating in the SEM images of our biofilms (Figure 6). Comparisons of sanitizers should be applied by a standardized regimen with sufficiently robust biofilms that can readily distinguish differences between biocides.

Author Contributions: The authors have participated in the following: conceptualization, P.M.M.; methodology, P.M.M., and M.A.; validation, M.A..; formal analysis, P.M.M., and M.A.; investigation, M.A.; resources, P.M.M.; writing—original draft preparation, M.A..; writing—review and editing, P.M.M., and M.A.; visualization, P.M.M., and M.A.; supervision, P.M.M.; project administration, P.M.M.; funding acquisition, P.M.M.

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Conflicts of Interest: The authors declare no conflict of interest.

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Optimization of a Microplate Assay for Generating *Listeria Monocytogenes*, *E. Coli* O157:H7, and *Salmonella* Biofilms and Enzymatic Recovery for Enumeration

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Abstract: Biofilms enable the persistence of pathogens in food processing environments. Sanitizing agents are needed that are effective against pathogens entrapped in biofilms that are more difficult to inactivate than planktonic cells that are displaced and found on equipment surfaces. We examined conditions to develop, analyze, and enumerate the enhanced biofilms of three different foodborne pathogens assisted by fluorescence adherence assay and enzymatic detachment. We compared three different isomeric forms of fluorescent substrates that are readily taken up by bacterial cells based on carboxy-fluorescein diacetate (5-CFDA, 5,6-CFDA, 5,6-CFDA, SE). Biofilm-forming strains of *Escherichia coli* O157:H7 F4546 and *Salmonella* Montevideo FSIS 051 were identified using a microplate fluorescence assay defined previously for *L. monocytogenes*. Adherence levels were determined by differences in relative fluorescence units (RFU) as well as recovered bacterial cells. Multiple hydrolytic enzymes were examined for each representative pathogen for the most suitable enzyme for detachment and enumeration to confirm adherence data obtained by fluorescence assay. Cultures were grown overnight in microplates, incubated, washed and replenished with fresh sterile growth medium; this cycle was repeated for seven consecutive days to enrich for robust biofilms. Treatments were performed in triplicate and compared by one-way analysis of variance (ANOVA) to determine significant differences ($p < 0.05$).

Keywords: biofilm; carboxyfluorescein diacetate; *Listeria monocytogenes*; *Salmonella*; *E. coli* O157:H7; microplate assay; enzymes

1. Introduction

The development of a biofilm usually involves a cellular adherence event that develops into irreversible attachment followed by development of a 3-dimensional biofilm that progresses into a mature and intricate biofilm [1,2]. During this progression, individual cells or portions of biofilm may slough off that are distributed elsewhere [3]. The initial or reversible attachment of planktonic cells to surfaces involves hydrophilic/hydrophobic interactions whereas the subsequent irreversible attachment may be due to the development of stronger covalent bonds as demonstrated by bacterial lectins and fimbriae [4–6]. Attachment is affected by the physiochemical properties of the surface, hydrodynamics, bacterial properties, and may also involve quorum sensing [7]. After attachment, micro-colonies are rapidly formed and the secretion of extracellular polysaccharides (EPS) start to develop, becoming the 'glue' of the biofilm architecture. As biofilms mature, higher densities of EPS, channels, and pores results in the positioning of bacteria away from the substrate surface and facilitates

the release of planktonic cells or sloughing off as displaced biofilm particles [8]. This can occur due to environmental shear forces, fluid dynamics, or abrasion [7]. Biofilms are generally problematic wherever they are found and may cause biofouling on the bottom of boats [9], in plumbing systems [10], on medical devices (intravenous catheters) and dental surfaces (plaque) [11,12]. Biofilms may also be involved in adverse health consequences when found on equipment surfaces in food manufacturing facilities [13–15].

Methods used to quantify bacterial adherence on surfaces have ranged from crystal violet staining (microscopic view) or absorbance readings [16,17] to sophisticated modern methods using 3-dimensional non-destructive analysis [18]. An in-situ fluorescence assay to assess the relative degree of attached bacteria has been implemented based on flow cytometry technique whereby individual fluorescing cells are quantified as they pass a laser beam. This procedure was used to screen adherence properties of numerous strains of *Listeria monocytogenes* [19]. Many strategies have been developed to disperse biofilms, as they pose a threat in food industries, dairy plants, prosthetic devices, human health (plaque) and many other areas [7,20,21]. The use of hydrolytic enzymes has been used in sanitation regimens to detach or disintegrate biofilms found in food processing plants [19,22]. Because of the repeated occurrence of in-plant contamination problems with *L. monocytogenes*, *E. coli* O157:H7, and *Salmonella* serovars, we examined improvements to a method to create robust biofilms with hardy biofilm formers to facilitate biofilm research. The current work examined multiple strains of three pathogens, fluorescent substrates, and hydrolytic enzymes to recover and enumerate these bacteria from biofilms.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

A variety of *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* serovars and strains from our culture collection were screened by a microplate fluorescence adherence assay to confirm or identify high level adherence. Strongly-adherent strains were then further used to optimize biofilm formation and enzyme detachment (enumeration recovery) assays such that they could be used for the evaluation of sanitizers in subsequent studies. Cultures were stored frozen by centrifuging 9 mL of overnight culture and resuspending cell pellets in 2–3 mL of fresh sterile Brain Heart Infusion (BHI, Difco, Becton-Dickenson, Franklin Lakes, NJ, USA) broth containing 10% glycerol. Cell suspensions were then stored in glass vials in an ultra-low freezer (−80 °C). Frozen stocks were thawed and revived by transferring 100 µL into 9 mL of BHI, incubating overnight at 30 °C, and sub-culturing at least twice before use in assays. Microbial enumeration for all the assays was carried out on Tryptic Soy Agar (TSA, Difco) plates, plated in duplicate. Although we screened a variety of strains in our culture collection, the main organisms used in this study were: *L. monocytogenes* 99-38, isolated from ground beef [19], *E. coli* O157:H7 F4546, an outbreak isolate from alfalfa sprouts [23,24], and *Salmonella* Montevideo FSIS 051, an isolate from beef [25].

2.2. Microplate Adherence Assay

The fluorescence microplate adherence assay has been used as a qualitative screening measure to identify strongly-adherent bacteria. This method was previously used to identify adherence properties of numerous strains of *L. monocytogenes* isolated from raw and ready-to-eat (RTE) meat processing plants in which *L. monocytogenes* 99-38 was identified as a strongly-adherent strain [19,26,27]. The adherence of *L. monocytogenes* 99-38 (strongly-adherent) and CW 35 (weakly adherent) were confirmed in this study as a control for the 5,6-CFDA fluorescence adherence assay used to screen *E. coli* and *Salmonella* spp. Strains of *Salmonella* spp. obtained from the United States Department of Agriculture, Agricultural Research Service [28], and strains of *E. coli* O157:H7 obtained from S.E. Gilliland [29] were screened to identify strongly-adherent strains that could serve as good biofilm formers.

Various parameters were then tested on biofilms of select pathogens grown in microplates such as type of fluorescent dye, number of washes, age of biofilms, and enzymatic release of attached cells for microbial enumeration before settling on a standardized assay prior to use in testing sanitizers against biofilms produced by these organisms.

2.2.1. Fluorescence Substrate for Fluorescence Microplate Assay

The single-isomer substrate 5-carboxyfluorescein diacetate (5-CFDA) and mixed-isomer substrates 5,6-carboxyfluorescein diacetate (5,6-CFDA) and 5,6-carboxyfluorescein diacetate, succinimidyl ester (5,6-CFDA, SE; Molecular Probes/Invitrogen, Carlsbad, CA, United States) were compared for the ability to produce fluorescence signals in a microplate biofilm assay and hence to determine which one was a more suitable substrate for our application. The fluorescent dyes were dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich, St. Louis, MO, USA) to get 2% (*w/v*) stock solutions. Working solutions were prepared thereafter by allocating 10 µL of the stock solutions to 1 mL of Tris buffer (0.05 M, pH 7.4). The best performing fluorophore above was incubated with serial dilutions of planktonic cells of *L. monocytogenes* 99-38 to determine if the fluorophore would be overwhelmed by high cell levels that are likely to be present in extended biofilms. Fluorescence emission was read in a Tecan GENios plate reader (Phenix Research Products, Hayward, CA, USA) using a fixed signal gain of 75% with excitation at 485 nm and detection at 535 nm and results were expressed as relative fluorescence units (RFU) [19,30].

2.2.2. Microplates as a Substrate for Attachment and Biofilm Formation

Black, non-treated 96-well flat-bottomed microplates (Cat: 237105, NUNC, Roskilde, Denmark) were used to perform fluorescence assays with adhered bacteria. Black plates prevent "cross-talk" from neighboring wells during fluorescence measurement and fluorescence signals can be read from the top. When fluorescence was not needed, sterile Falcon 96-well clear, non-treated flat-bottomed polystyrene microplates (Cat: 351172, Corning Inc., Corning, NY, USA) were used to grow microbial biofilms and perform subsequent washing, enzyme detachment, and enumeration assays.

2.2.3. Microplate Washing

The microplates used for fluorescence or biofilm enumeration assays, were subjected to a wash treatment in a Biotek Elx405 Magna plate washer (Ipswich, Suffolk, UK). This microplate washer was connected to separate liquid supply containers of 10% Clorox disinfecting bleach solution (Clorox Co., Oakland, CA, USA), sterile de-ionized water, 0.05 M Tris buffer (pH 7.4), and additional waste containers. The plate washer has 96 pairs of needles (a longer one for aspiration and a shorter one for dispensing) to draw liquids into, and out of, each of the wells and a shake parameter to shake the plate to re-suspend settled cells or release loosely adhered cells before washing. Maintenance cycles were performed to sanitize the plate washer needles and tubing by washing with 10% Clorox bleach solution (2 times), followed by sterile de-ionized water (3 times), and sterile Tris buffer (2 times) before and after use with biofilm-containing plates.

In order to determine how many washes were sufficient to remove loosely adhered cells from microplates prior to enzymatic treatment, we set up a series of plates that would be washed 1–4 times with 0.05 M Tris buffer (pH 7.4) using the 'shake' option in the Elx405 plate washer during each wash. After each wash series, buffer was added manually to microplates, shaken for 10 s, and then recovered and plated for enumeration of planktonic cell counts.

2.3. Enzymatic Detachment of Adhered Cells from Microplates

A variety of enzymes were used that act on different biochemical substrates that may be involved with attachment to surfaces. Previously, we examined similar enzymes for the ability to release *L. monocytogenes* [19]; in this study, we examined a similar set of enzymes for ability to release *L. monocytogenes* 99-38, *E. coli* O157:H7 F4546, and *Salmonella* Montevideo FSIS 051.

2.3.1. Enzymes for Microbial Detachment

Bax protease (DuPont Qualicon, Wilmington, DE, USA) was obtained as a premade solution and used as per manufacturer's guideline [12.5 µL in 1 mL Tris buffer (0.05 M, pH 7.4)] [19]. The specific protease and concentration/activity is undisclosed as it is a proprietary solution for their PCR kit.

Pronase E (P5147, Sigma-Aldrich, St. Louis, MO, USA) powder from *Streptomyces griseus* (5.3 U/mg) was prepared in Tris buffer (0.05 M, pH 7.4) to obtain a stock solution of 500 U/mL.

Trypsin (T4549, Sigma-Aldrich) from porcine pancreas was obtained in liquid form (1486 U/mL) and was diluted with Tris buffer (0.05 M, pH 7.4) to 500 U/mL.

Papain (5125, EMD Millipore Corp., Billerica, MA, USA) from *Carica papaya* (31,850 U/mg) was prepared in Tris buffer (0.05 M, pH 7.4) to a concentration of 1000 U/mL.

Cellulase (C1184, Sigma-Aldrich, 1.3 U/mg) powder from *Aspergillus niger* (1.3 U/mg) was added to Tris buffer (0.05 M, pH 7.4) to get a desired working stock solution of 500 U/mL.

Lipase (L1754, Sigma-Aldrich) powder from *Candida rugosa* (1170 U/mg) was dissolved in Tris buffer (0.05 M, pH 7.4) to get a working stock solution of 500 U/mL.

Except for the commercially-obtained Bax protease, all of the enzyme solutions were filter-sterilized via 0.22 µm filters and held in the refrigerator or stored frozen (−20 °C) if not used within 1–2 days.

2.3.2. Enzymatic Detachment and Enumeration Assay

The enzymes were evaluated against biofilms of the 3 pathogenic genera to determine which would be the best for recovering bacterial cells from biofilms. Overnight cultures (~9-log CFU/mL) of the three most strongly-adherent pathogenic microbes (one strain from each of the three genera) were diluted to ~4-log CFU/mL in BHI broth. A 200 µL aliquot of each culture was allocated, in triplicate, into Falcon 96-well microplates; each replication of the same organism used a separately-inoculated culture. The microplates were sealed with Parafilm (Fisher Scientific, Waltham, MA, USA) to avoid evaporation and incubated at 30 °C for 24 h. After that, the microplates were washed 3 times with Tris buffer (0.05 M; pH 7.4) in a Biotec Elx405 Magna plate washer as described earlier. A 'shaking' option was used to re-suspend settled planktonic cells and loosely held cells. This was followed by the addition of fresh BHI (200 µL) into the wells and an additional incubation for 24 h at 30 °C. The same process of washing with Tris buffer and adding fresh BHI into wells was repeated each day for one week. After 7 days of washing and incubating, the final wash with Tris buffer using the plate washer (with shaking) was performed and 200 µL of different enzymes at the earlier stated concentrations were transferred into the experimental wells. After the addition of enzymes, the microplate was incubated for 1 hour at 37 °C. Finally, to get detached cell counts, the solutions from the wells were further diluted and plated on Tryptic Soy Agar (TSA) plates and incubated at 30 °C for 24–36 h.

2.4. Statistical Analysis

Each trial was performed in triplicate replication where each replication was treated as an independent and autonomous experiment using separately inoculated cultures and prepared plating media. All data were presented as the mean of triplicate replications and standard deviation of the mean were represented by error bars. Statistical analysis was done by using one-way analysis of variance (ANOVA) and Holm-Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$). Data bars with different letters are significantly different ($p < 0.05$); data with the same letters are not significantly different ($p > 0.05$).

3. Results

3.1. Choice of Fluorescent Substrate

We obtained significantly lower levels of fluorescence using 5,6-CFDA, SE and 5-CFDA compared to fluorescence obtained with 5,6-CFDA (Figure 1A). The 5,6-CFDA fluorophore was also examined for whether the level used was limiting when using high levels of bacterial cells as observed during enzymatic detachment. When 2-fold dilutions of planktonic bacteria (~9-log CFU/mL) were incubated with the same amount of 5,6-CFDA as used in adherence assays, no loss of signal linearity was observed (Figure 1B).

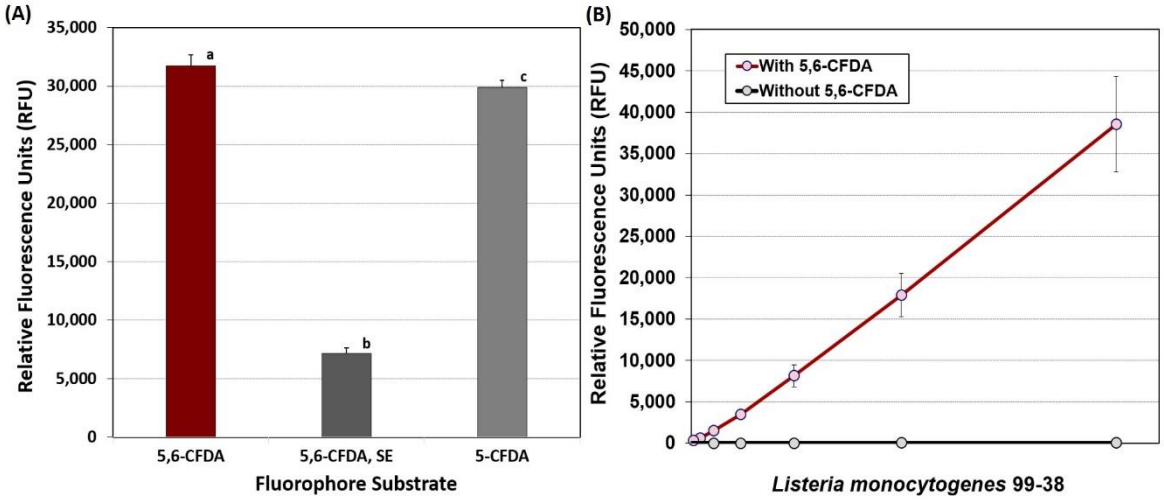

Figure 1. (**A**) Comparison of fluorescence signals obtained using *L. monocytogenes* 99-38 in microplate fluorescence assay with 5,6-CFDA, 5,6-CFDA, SE, or 5-CFDA. Data are presented as the mean of triplicate replications and error bars represent the standard deviation from the mean. Means with different letters are significantly different as determined by one-way ANOVA using the Holm-Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$). (**B**) Two-fold dilutions of planktonic cells incubated with 5,6-CFDA compared to cells without 5,6-CFDA and examined for fluorescence signals (Ex/Em: 485/535 nm). Error bars are the standard deviation of the means of triplicate replications.

3.2. Screening of Listeria monocytogenes, Salmonella, and E. coli O157:H7 via Fluorescence Microplate Assay

The adherence characteristics of numerous strains of *L. monocytogenes* isolated from ready-to-eat meat processing facilities had previously been examined [27]. In this study, we again confirmed that *L. monocytogenes* 99-38 was a strongly-adherent strain in comparison with weakly-adherent *L. monocytogenes* CW35 (Figure 2A).

Fluorescence microplate assays of various serovars of *Salmonella* from our culture collection demonstrated that *Salmonella* Montevideo FSIS 051 was the most adherent strain and could be useful in development of microplate *Salmonella*-based biofilms to be challenged with various sanitizers in a convenient microplate format (Figure 2B).

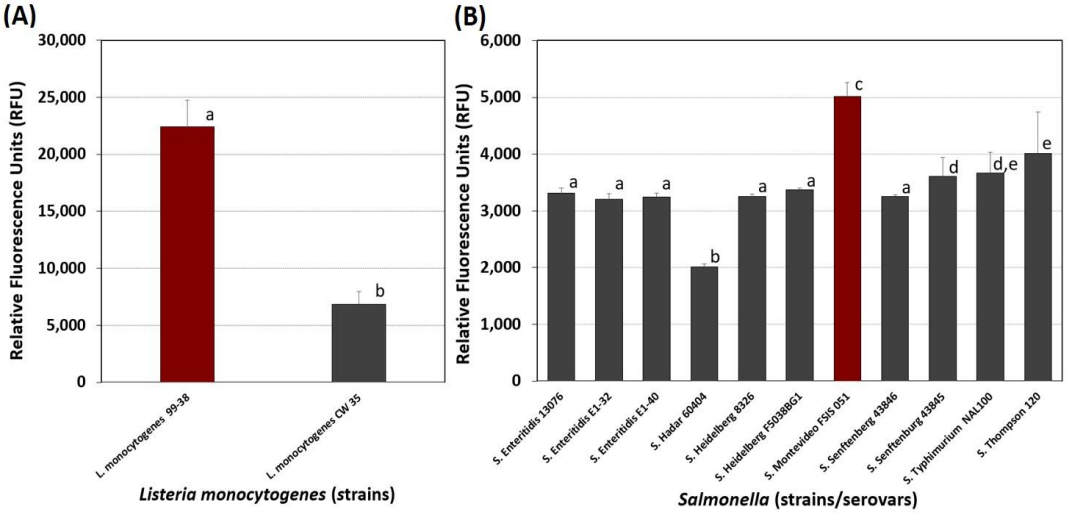

Figure 2. Comparison of adherence of (**A**) *Listeria monocytogenes* and (**B**) *Salmonella* by microplate fluorescence assay with 5,6-CFDA. Data are presented as the mean of triplicate replications and error bars represent the standard deviation from the mean. Means with different letters are significantly different as determined by one-way ANOVA using the Holm-Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$); means with the same letter are not significantly different ($p > 0.05$).

We also examined a variety of *E. coli* O157:H7 strains isolated from cattle trucks and a known biofilm forming *E. coli* O157:H7 strain (F4546) to screen for strong adherence using the fluorescent adherence assay (Figure 3). Although some strains were moderately adherent (K-3995, K-4492), none were as strongly adherent as the *E. coli* O157:H7 F4546 control strain that is known for adherence (Figure 3)

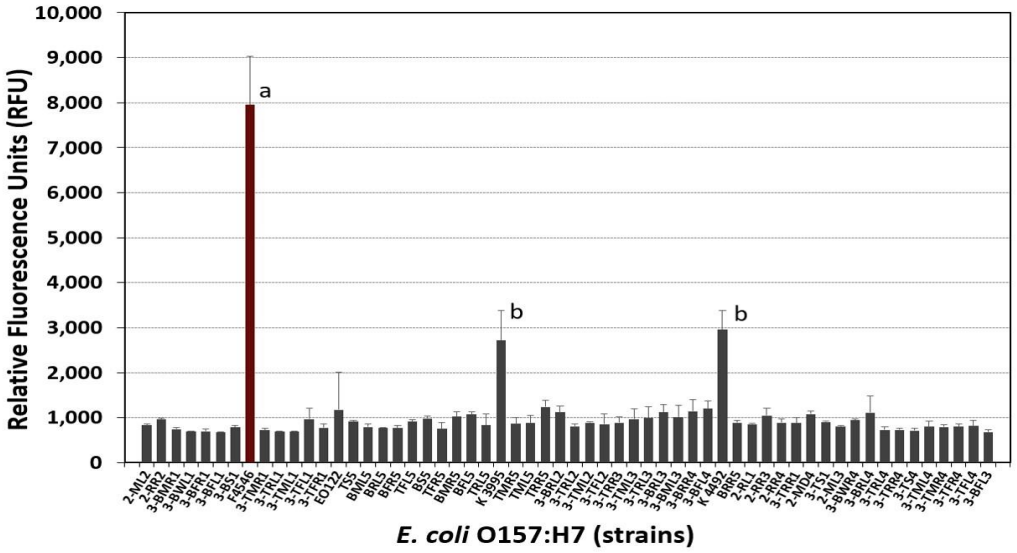

Figure 3. Comparison of adherence of various strains of *E. coli* O157:H7 by microplate fluorescence assay with 5,6-CFDA. Data are presented as the mean of triplicate replications and error bars represent the standard deviation from the mean. Means with different letters are significantly different as determined by one-way ANOVA using the Holm-Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$); means with the same letter are not significantly different ($p > 0.05$).

3.3. Buffer Washes with Microplate Biofilms

Biofilms of *L. monocytogenes* were examined for a number of washes required to remove 'loosely retained' cells prior to enzymatic treatment for biofilm enumeration. When subjected to 4 rounds of buffer washes using an automated 'shaking step', we found that 3 washes were sufficient to remove loose cells and further washes did not further diminish the levels of cells that are leaching from the biofilm (Figure 4).

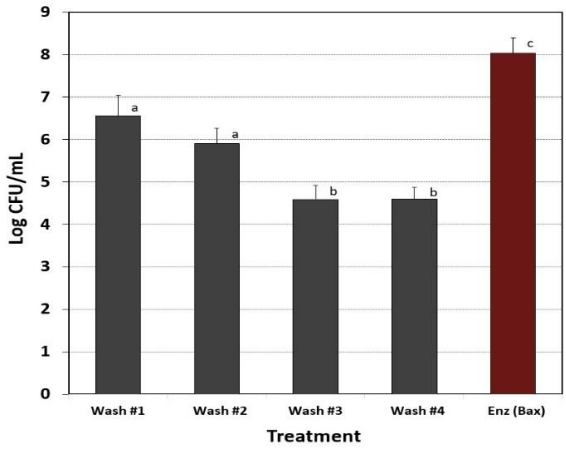

Figure 4. Enumeration of viable cells after multiple buffer washes with 0.05 M Tris buffer (pH 7.4) and after Bax protease treatment (after final wash) of *L. monocytogenes* 99-38 microplate biofilms. Data are presented as the mean of triplicate replications and error bars represent the standard deviation from the mean. Means with different letters are significantly different as determined by one-way ANOVA using the Holm-Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$); means with the same letter are not significantly different ($p > 0.05$).

A side-by-side comparison was also made of the 3 pathogens selected from the prior screening efforts: *L. monocytogenes* 99-38, *E. coli* O157:H7 F4546, and *S.* Montevideo FSIS 051 using the same wash procedure and Bax protease enumeration method (Figure 5A) while using the fluorescent substrate assay to examine levels before and after recovery from microplates (Figure 5B).

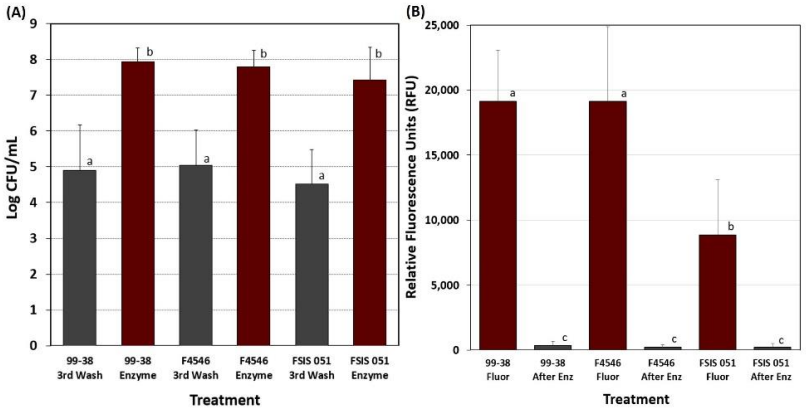

Figure 5. Comparison of enumeration and fluorescence data with *L. monocytogenes* 99-38, *Salmonella* Montevideo FSIS 051, and *E. coli* F4546, before and after enzyme treatment of microplate biofilms. (**A**) Cell enumeration after 3rd round wash buffer followed by Bax protease release of adhered cells from microplates. (**B**) Fluorescence of biofilms with 5,6-CFDA before and after Bax protease treatment to release bacterial cells. Data are presented as the mean of quadruple replications and error bars represent the standard deviation from the mean. Significant differences are between treatments with the same strain. Means with different letters are significantly different as determined by one-way ANOVA using the Holm-Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$).

3.4. Development of Extended Biofilms in Microplates

In previous studies, a short 2–3 day cycle of repeated microplate washing/incubation was used to establish microplate biofilms [19]. In this study, conditions were sought that would achieve a robust 7-day biofilm for use in upcoming studies to examine the effect of sanitizers on biofilms of foodborne pathogens. This was done by repeated cycles of growth, adherence, washing away of planktonic/loose cells, and addition of sterile media to continue growth from those cells attached to microplate surfaces. In prior studies, several enzymes were used to recover and enumerate attached bacterial cells from microplates such as pronase E (Figure 6) and Bax protease [19]. The data shows that we were able to incrementally increase the level of adhered cells by >12-fold over 7-days using the 'extended biofilm' approach to create a more robust and challenging biofilm (Figure 6).

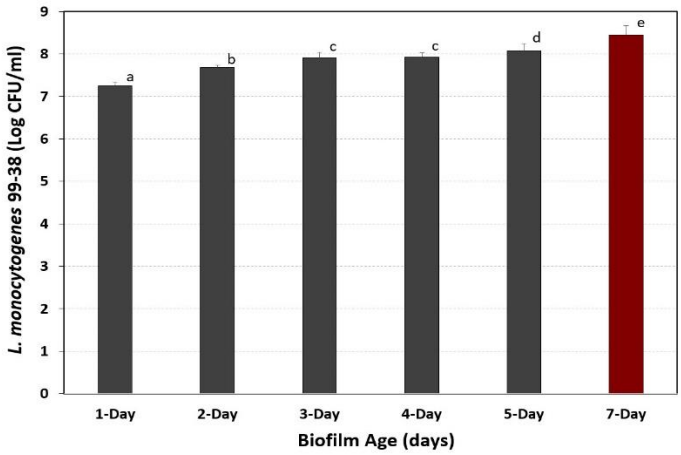

Figure 6. Enumeration of *L. monocytogenes* 99-38 biofilm levels over time after repeated incubation in microplates. Planktonic cells were removed daily, washed with buffer, and replaced with fresh sterile media. Attached cells were enumerated by detachment with pronase E and represented as the means of triplicate replications; error bars represent the standard deviation of the means. Means with different letters are significantly different as determined by one-way ANOVA using the Holm-Sidak test for pairwise multiple comparisons to determine significant differences ($p < 0.05$); means with the same letter are not significantly different ($p > 0.05$).

3.5. Evaluation of Various Enzymes for Bacterial Detachment and Enumeration

In prior assays, both Bax protease [19] (Figures 4 and 5) and pronase E (Figure 6) were used for detaching cells from biofilms for enumeration. Although Bax protease was effective, the identity of the particular protease in this proprietary commercial reagent was unknown. It was also important to re-evaluate which enzyme(s) worked best to enumerate cell levels from microplate assays with *L. monocytogenes*, *S.* Montevideo, and *E. coli* O157:H7 biofilms in upcoming studies. Although all the enzymes tested worked sufficiently well to obtain high level cell counts from 7-day biofilms, three were nearly equal for both *L. monocytogenes* 99-38 and *E. coli* O157:H7 F4546, including Bax protease, pronase E, and trypsin (Figure 7). However, for *Salmonella*, trypsin was significantly better than the other enzymes (Figure 7).

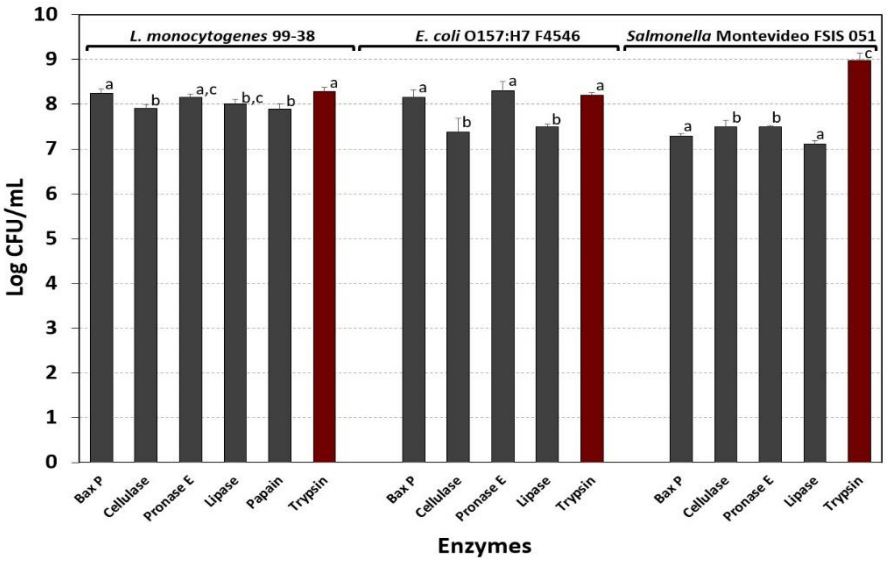

Figure 7. Recovery and enumeration of *L. monocytogenes* 99-38, *E. coli* O157:H7 F4546, and *Salmonella* Montevideo FSIS 051 biofilms after treatment with various enzymes (Bax protease, cellulase, pronase E, papain, trypsin, or lipase). Data bars represent the means of triplicate replications and error bars represent standard deviation of the means. Means with different letters are significantly different as determined by one-way ANOVA using the Holm-Sidak test for pairwise multiple comparisons within the same organism to determine significant differences ($p < 0.05$); means with the same letter are not significantly different ($p > 0.05$).

4. Discussion

Biofilms have been measured in various ways, including absorbance measurements taken directly with a plate reader from biofilms in microplates stained with crystal violet [31,32], or indirectly from dye recovered by de-staining solutions [33,34], after vortexing with glass beads to recover cells for enumeration [35] or after enzyme treatment and sonication to dislodge cells [36]. Microplate adherence and fluorescence visualization was examined to mimic flow cytometry whereby individual cells are 'counted' based on fluorescence of internalized fluorophores as they pass through a capillary tube that is transected by a laser. In our work, this method was modified for assessment of adherent cells attached in situ to the walls of microplate wells using a fluorescence plate reader as a qualitative and quick screening procedure.

In prior and current studies, 'non-treated' microplates were used to assess the bacterial strains' inherent and unaided ability to adhere (i.e., 'treated' plates are often used with tissue culture assays to promote adherence). It is likely that treated plates could subsequently be used to provide even more formidable biofilms for use in challenge studies with antimicrobials and sanitizers. Flat-bottom microplates are also important when using microplate washers as those with curved bottoms might interfere with lowering of the paired needles of the plate washer into the plates and/or leave significant amounts of wash fluid behind.

Fluorescein is a fluorescing compound that can freely diffuse through cell membranes. Its use became popularized by applications in flow cytometry [37], vital staining of live/dead bacterial cells [38], and applications allowing visualization by microscopy and studies in apoptosis [39]. Unmodified fluorescein can fluoresce externally when applied to bacterial cells whereas the signal can be quenched by modification with diacetate (i.e., 5,6-CFDA). Once internalized, cytoplasmic esterases hydrolyze the fluorophore, causing a significant spike in intracellular fluorescence. The purported benefit of the succinyl ester of 5,6-CFDA (5,6-CFDA, SE) was that in addition to diacetate, the -SE modification allows it to be retained longer intracellularly because of the propensity to bind to amino groups. Fuller et al. [40] used 5,6-CFDA,SE to follow the fate of labelled bacterial cells under no growth conditions for 28 days in groundwater sediment microcosms as they retained fluorescence during

this time period. We contemplated whether this feature would allow us to achieve higher and more sensitive detection levels. However, 5,6-CFDA not only performed better than 5-CFDA or 5,6-CFDA, SE (Figure 1A), but was also significantly less expensive than the other fluorophores. The use of 5,6-CFDA in our application has been to qualitatively indicate the degree of adherence of cells as in a biofilm when screening numerous strains. When 5,6-CFDA was mixed with serial dilutions of planktonic cells, we observed a linearity of signal even with the least diluted (highest) level of cells suggesting that the levels used in our biofilm assays were not limiting (Figure 1B). The fact that biofilm-adhered cells are possibly diffusion limited compared to planktonic cells further suggests that levels of fluorophore are not limiting in our biofilm assays.

In establishing conditions for *Listeria, Salmonella,* and *E. coli* biofilms, *L. monocytogenes* 99-38 was confirmed [19] as a robust, strongly-adherent strain that would serve well in the formation of biofilms for challenge studies (Figure 2A). Strains of *E. coli* O157:H7 and *Salmonella* in our collection were further screened in search of good biofilm formers in preparation of a project to evaluate the effect of sanitizers on biofilms of *L. monocytogenes, Salmonella* spp., and *E. coli* O157:H7. Among the *Salmonella* serovars in our collection, *Salmonella* Montevideo FSIS 051 was significantly more adherent than the other strains tested (Figure 2B). *E. coli* F4546, a strain implicated in biofilm formation [41], was also the most adherent from among the 58 strains of *E. coli* O157:H7 tested (Figure 3). Based on the fluorescent microplate data with these strains, we continued optimization of biofilm adherence with *L. monocytogenes* 99-38, *Salmonella* Montevideo FSIS 051, and *E. coli* F4546 (Figures 2 and 3).

Microplate biofilms comprised of *L. monocytogenes* 99-38 were washed multiple times with buffer before enzymatic treatment to remove the bacterial culture media, planktonic, and loosely adhered cells. The plate washer had a built-in 'shake' mode that provided a standardized shaking regimen to release loosely held cells, but it is possible that this process will always free up cells. It was decided that as the level of cells stabilized (i.e., after 2–4 washes), that this was indicative of a sufficient degree of washing (Figure 4). The chosen wash parameter was examined for all 3 of the selected strains (99-39, FSIS 051, and F4546) to insure that loosely held cells were removed (Figure 5A). Although high levels of cells were still recovered in the wash buffer, they represented a small proportion of the levels attached to the well surfaces (Figure 5A). Similar 'before and after' trials using fluorescence assays were performed with a full biofilm load and were compared to similar wells after detachment of cells with protease, washing with buffer, and application of fluorescent substrate (Figure 5B). The absence of significant detectable fluorescent signal after enzyme treatment supports the data obtained with enumeration after enzymatic detachment. The application of 5,6-CFDA does not affect the viability of cells, and therefore wells treated with 5,6-CFDA could be used directly after fluorescence measurements for enzyme treatment and microbial platings.

Bacillus isolates from a dairy processing facility were recently shown to produce thick biofilms that were resistant to routine clean-in-place procedures [42]. Similarly, our intention was to increase the robustness of our biofilms so that they would be a better challenge in upcoming studies evaluating commercial sanitizers as is being done by others [43]. We did this by selecting strains that were superior in their initial adhesion to surfaces, and then used a daily cycling of growth in media, buffer wash, fresh media, and further incubation that was repeated for 7 days to provide a more robust biofilm. Microplate biofilms were examined in 1-to-7-day increments (i.e., different plates for the respective days) by enzymatic detachment and enumeration of cell levels to insure that incremental increases were occurring during the extended cycle times (Figure 6). Since enzymatic recovery of biofilms from *E. coli* or *Salmonella* were not examined previously, we re-examined the application of hydrolytic enzymes for all 3 organisms in microplate biofilm assays. Bax protease, pronase E, and trypsin enumerated comparable levels of detached cells for *L. monocytogenes* 99-38 and *E. coli* F4546 (Figure 7). However, trypsin was significantly better in providing higher counts than the other enzymes with *Salmonella* Montevideo FSIS 051 and was the enzyme of choice going forward (Figure 7). Trypsin has long been used in tissue culture studies for releasing tissue culture cells from flasks [44], and it is the least expensive of the enzymes used in this study and is sold in a convenient liquid form.

It is becoming more evident that biofilms are among the root causes of many recurring foodborne illness outbreaks and spoilage contaminations [45], and enzymes are being further exploited to address them [46]. The complex heterogeneity in the composition of some biofilms may ultimately require unique biological enzymes [47,48] or combinations of enzyme and chemical treatment [49] to eradicate troublesome biofilms.

5. Conclusions

This improved microplate biofilm assay will be useful for enumerating initial and residual viable cells in studies on biofilms of *L. monocytogenes*, *Salmonella*, and/or *E. coli* O157 that currently present recurring problems in food processing facilities. Trypsin and 5,6-CFDA are both the most effective and least expensive of the alternative components we examined. Bacteria continue to leach from such biofilms, and mimics similar situations in food processing facilities where potential biofilms on food contact surfaces may contaminate passing foods as foci of contamination by the sloughing off of loosely-held cells. We hope to examine the effect of commercial sanitizers using these challenge organisms to identify those that might be more effective against biofilms.

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