1   *Article*

2 Optimization of an Extended Microplate Assay for
3 Generating *Listeria monocytogenes*, *E. coli* O157:H7,
4 and *Salmonella* Biofilms and Enzymatic Recovery for
5 Enumeration

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11

12   **Abstract:** Biofilms enable the persistence of pathogens in food processing environments. In order
13   to inactivate these microorganisms, sanitizing agents are needed that are effective against pathogens
14   entrapped in biofilms which are more difficult to inactivate than planktonic cells that are displaced
15   and found on equipment surfaces. We examined conditions to develop, analyze, and enumerate
16   robust biofilms of 3 different foodborne pathogens assisted by fluorescence adherence assay and
17   enzymatic detachment. We compared 3 different isomeric forms of fluorescent substrates that are
18   readily taken up by bacterial cells based on carboxy-fluorescein diacetate (5-CFDA, 5,6-CFDA, 5,6-
19   CFDA,SE). Biofilm-forming strains of *L. monocytogenes*, *E.coli* O157:H7, and *Salmonella* serovars were
20   detected using the chosen substrate in a microplate fluorescence assay define previously for use
21   with *Listeria*. Adherence levels were determined by differences in relative fluorescence units (RFU).
22   Multiple hydrolytic enzymes were examined for each representative pathogen for the most suitable
23   enzyme for detachment and enumeration to confirm data obtained by fluorescence assay. Cultures
24   were grown overnight in microplates, incubated, washed and replenished with fresh sterile growth
25   medium; this cycle was repeated 7 times before used as 'biofilms'. All treatments were performed
26   in triplicate and compared by one-way analysis of variance (ANOVA) to determine significant
27   differences ($p < 0.05$). Analysis of 7-day biofilms by SEM indicated possible extracellular
28   polysaccharide involvement with *Salmonella* and *E. coli.*

29   **Keywords:** Biofilm, carboxyfluorescein diacetate, *Listeria monocytogenes*, *Salmonella*, *E. coli* O157:H7,
30   microplate assay.

31

32 1. Introduction

33   The development of a biofilm usually involves a cellular adherence event that develops into
34   irreversible attachment followed by development of a 3-dimensional biofilm that progresses into a
35   mature and intricate biofilm [1]. During this progression, cells or portions may slough off that are
36   distributed elsewhere [2]. The initial or reversible attachment of planktonic cells to surfaces involves
37   hydrophilic/hydrophobic interactions whereas the subsequent irreversible attachment is due to the
38   development of stronger covalent bonds [3]. Attachment is affected by the physiochemical properties

39 of the surface, hydrodynamics, bacterial properties, and may also involve quorum sensing [4]. After
40 attachment, micro-colonies are rapidly formed and the secretion of extracellular polysaccharide (EPS)
41 starts to develop, becoming the 'glue' of the biofilm architecture. As biofilms mature, higher densities
42 of EPS, channels, and pores results in the positioning of bacteria away from the substrate surface and
43 facilitates release of planktonic cells or sloughing off as displaced biofilm particles [5]. This can occur
44 due to environmental shear forces, fluid dynamics, or abrasion [4]. Biofilms are generally problematic
45 wherever they are found and may cause biofouling on the bottom of boats [6], in plumbing systems
46 [7], on medical devices (intravenous catheters) and dental surfaces (plaque) [8,9]. Biofilms may also
47 be involved in adverse health consequences when found on equipment surfaces in food
48 manufacturing [10,11].

49   Traditional methods used to quantify bacterial adherence on surfaces have included crystal
50 violet staining (microscopic view) or absorbance readings [12,13]. An *in situ* fluorescence assay to
51 assess the relative degree of attached bacteria has been implemented based on flow cytometry
52 technique whereby individual fluorescing cells are quantified as they pass a laser beam. The
53 procedure was used to screen adherence properties of various strains of *Listeria monocytogenes* [14].
54 Many strategies have been developed to disperse biofilms, as they pose a threat in food industries,
55 dairy plants, prosthetic devices, human health (plaque) and many other areas [4,15,16]. The use of
56 proteolytic enzymes has been suggested to detach or disintegrate biofilms [14,17]. Cellulases, lipases
57 and proteases have been used to detach adhered *L monocytogenes* cells from different surfaces. The
58 detachment of cells was enumerated by plating cells after enzyme treatment of biofilm coated wells.
59 The recovered cell counts helped quantify the detached cells. Maximum detachment of cells was
60 obtained with BAX-protease enzyme [14]. In the current work we used repeated microplate culture
61 to develop a challenging biofilm to facilitate future studies on sanitizer/antimicrobial effectiveness
62 and the use of enzymatic detachment to readily allow bacterial enumeration of biofilms created with
63 *Listeria monocytogenes*, *E. coli* O157:H7, and *Salmonella*.

64 **2. Materials and Methods**

65 *2.1. Bacterial strains and growth conditions.*

66   A variety of *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* serovars and strains from our
67 culture collection were screened by a microplate adherence screening assay to confirm or identify
68 high level adherence. Select, strongly-adherent strains from each genus were then further used for
69 optimization of biofilm formation and enzyme detachment (enumeration recovery) assays such that
70 they could be used for evaluation of sanitizers in subsequent studies. Cultures were stored frozen by
71 centrifuging 9 ml of overnight cultures and re-suspending the pellets in 2-3 ml of fresh sterile BHI
72 broth containing 10% glycerol and then stored in glass vials in an ultra-low freezer (-80°C). The frozen
73 stocks were thawed and revived by transferring 100 µl into 9 ml of Brain Heart Infusion (BHI) broth.
74 The BHI tubes with cultures were then incubated overnight at 30° C and sub-cultured at least twice
75 before use in assays. Microbial enumeration for all the assays was carried out on Tryptic Soy Agar
76 (TSA) plates, plated in duplicate. Although we screened a variety of strains in our culture collection,
77 the main organisms used in this study were: *L. monocytogenes* 99-38, isolated from ground beef [14],
78 *E. coli* O157:H7 F-4546, an outbreak isolate from alfalfa sprouts [18], and *Salmonella* Montevideo FSIS
79 051, an isolate from beef [19].

80 *2.2. Microplate adherence assay.*

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

85 2.2.1 Fluorescence substrate.

86 The single-isomer substrate 5-carboxyfluorescein diacetate (5-CFDA) and mixed-isomer substrates
87 5,6-carboxyfluorescein diacetate (5,6-CFDA) and 5,6-carboxyfluorescein diacetate, succinimidyl ester
88 (5,6-CFDA, SE; Molecular Probes/Invitrogen, Carlsbad, CA) were compared for the ability to produce
89 fluorescence signals in a microplate biofilm assay and hence to determine which one was a more
90 suitable substrate for our application. The fluorescent dyes were dissolved in dimethyl sulfoxide
91 (DMSO) to get 2% (w/v) stock solutions. Working solutions were prepared thereafter by allocating
92 10 μl of the stock solutions to 1 ml of Tris buffer (0.05 M, pH 7.4). The best performing fluorophore
93 above was incubated with serial dilutions of planktonic cells of *L. monocytogenes* 99-38 to determine
94 if the fluorophore would be overwhelmed by high cell levels that are likely to be present in extended
95 biofilms. Fluorescent signals were read in a Tecan GENios plate reader (Phenix Research Products,
96 Hayward, CA) using a fixed signal gain of 75% with excitation at 485 nm and detection at 535 nm.

97 2.2.2 Microplates as a substrate for attachment and biofilm formation.

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

104 2.2.3 Microplate washing.

105 The microplates used for detachment and lethality assays were subjected to a wash treatment in
106 a Biotek Elx405 Magna plate washer (Ipswich, Suffolk, United Kingdom). This microplate washer
107 was connected to separate liquid supply containers (10% bleach, sterile de-ionized water, or 0.05 M
108 Tris buffer, pH 7.4) and to additional waste containers. The plate washer has 96 pairs of needles (a
109 longer one for aspiration and a shorter one for dispensing) to draw liquids into, and out of, each of
110 the wells and a shake parameter to shake the plate to re-suspend settled cells, or to release loosely
111 adhered cells, before washing. Before washing the bacterial-adhered 96-well microplates,
112 maintenance cycles were performed to sanitize the plate washer needles and tubing by washing with
113 10% bleach (2 times), followed by sterile de-ionized water (3 times), and sterile Tris buffer (2 times).
114 In order to determine how many washes were sufficient to remove loosely adhered cells from
115 microplates prior to enzymatic treatment, we set up a series of plates that would be washed 1-4 times
116 with 0.05 M Tris buffer (pH 7.4) using the 'shake' option in the Elx405 plate washer during each wash.
117 After each wash series, buffer was added manually to microplates, shaken for 10 sec, and then
118 recovered and plated for enumeration of planktonic cell counts.

119 *2.3 Enzymatic detachment of adhered cells from microplates.*

120   A variety of enzymes were used that act on different microbial substrates that may be involved
121   with attachment to surfaces. Previously, we examined similar enzymes for ability to release *L.*
122   *monocytogenes* [14]; in this study we again examined a similar set of enzymes for ability to release *L.*
123   *monocytogenes* when attached to microplate surfaces, but also included *E. coli*, and *Salmonella*.

124   2.3.1 Enzymes for microbial detachment.

125   Bax protease (DuPont Qualicon, Wilmington, DE) was obtained as a premade solution and used
126   as per manufacturer's guideline [12.5 µl in 1 ml Tris buffer (0.05M, pH 7.4)] [14]. The specific protease
127   and concentration/activity is undisclosed as it is a proprietary solution for their PCR kit.

128   Pronase E (P5147, Sigma-Aldrich, St. Louis, MO) from *Streptomyces griseus* was obtained in
129   powdered form with activity of 5.3 U/mg. The working solution was prepared at concentration of 500
130   U/ml. For this purpose, 0.4715 gm of Pronase E powder was added to 5 ml of sterile Tris (0.05 M, pH
131   7.4), dissolved and filter sterilized (0.22 micron filter) to get a stock solution of 500 U/ml.

132   Trypsin (T4549, Sigma-Aldrich) from porcine pancreas was another protease enzyme used in
133   this research. It was obtained in liquid (solution) form with an activity of 1485.9 U/ml. We examined
134   trypsin at two concentrations, the original (1485.9 U/ml) and diluted to 500 U/ml.

135   Papain (5125, EMD Millipore Corp., Billerica, MA) from *Carica papaya* had a listed activity of
136   31,850 U/mg and a stock solution was prepared in 10 ml Tris (0.05 M, pH 7.4) at a concentration of
137   1000 U/ml.

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

142   Lipase (L1754, Sigma-Aldrich) from *Candida rugosa* had activity of 1,170 U/mg solid. Lipase
143   powder (2.14 mg) was dissolved in 5 ml of Tris (0.05 M, pH 7.4) to get a concentration of 500 U/ml
144   and filter (0.22 micron) sterilized.

145   2.3.2 Enzymatic detachment and enumeration assay.

146   In order to obtain a plate count enumeration of biofilm-adhered bacteria, either before (controls)
147   or after enzyme treatment (experimental), we had to evaluate and optimize the best method to detach
148   and recover viable cells. For this purpose, overnight cultures (~10^9 CFU/ml) of the three most strongly-
149   adherent pathogenic microbes (one from each of the three genera) were diluted to ~10^4 CFU/ml in
150   BHI broth. A 200 µl aliquot of each culture was allocated, in triplicate, into Falcon 96-well microplates.
151   The microplates were sealed with Parafilm (Fisher Scientific, Waltham, MA) to avoid evaporation
152   and then incubated at 30°C for 24 hours. After 24 hours, the wells were washed 3 times with Tris
153   buffer (0.05 M; pH 7.4) in a Biotec Elx405 Magna plate washer as described earlier. A 'shaking' option
154   was used to wash off loosely adherent cells in addition to re-suspending settled planktonic cells. This
155   was followed by the addition of fresh BHI (200 µl) into the wells and an additional incubation for 24
156   hours at 30°C. The same process of washing with Tris buffer and adding fresh BHI into wells was
157   repeated each day for one week. After 7 days of washing and incubating, the final wash with Tris
158   buffer using the plate washer (with shaking) was performed and 200 µl of different enzymes at the
159   earlier stated concentrations were transferred into the experimental wells. After the addition of
160   enzymes, the microplate was incubated for an hour at 37°C. Finally, to get detached cell counts, the

161 solutions from the wells were further diluted and plated on Tryptic Soy Agar (TSA) plates and
162 incubated at 30°C for 24-36 hours.

163 *2.4 Scanning electron microscopy (SEM) of biofilms.*

164 Biofilms of the strongly-adherent strains screened and confirmed by fluorescence microplate
165 assay were examined by scanning electron microscopy (SEM). The individual adherent strains *Listeria*
166 *monocytogenes* 99-38, *E. coli* O157:H7 F4546, and *Salmonella* Montevideo FSIS 051 were diluted in broth
167 media (~10^4 log cfu/ml in BHI media; 300 μL) and dispensed into Millicell EZ Slide 8 wells (Millipore
168 Sigma, Sheboygan Falls, WI), sealed with Parafilm (Fisher Scientific) to avoid evaporation, and
169 incubated at 30º C. The media (BHI) in the wells was aspirated, washed with Tris (0.05 M; pH 7.4),
170 and fresh sterile media added daily; this process was repeated for 7 days to get a 7-day old mature
171 biofilm in the wells. A standard protocol provided by Oklahoma State University's Electron
172 Microscopy lab was used to fix, dry and coat the samples before imaging. Cells were fixed for 2 h in
173 2.0% glutaraldehyde in 0.1 M cacodylate buffer (21.4 g sodium cacodylate brought to 500 ml with
174 dH_2O). The slides were then rinsed 3x in buffered wash (60 ml of 0.2 M cacodylate buffer, 140ml of
175 dH_2O, and 12.3g of sucrose; 15 min/rinse). Adherent cells were again fixed for 1 h in 1% aqueous
176 osmium tetroxide (OsO$_4$) at room temperature and then rinsed 3x in buffered wash solution (15
177 min/rinse). This was followed by dehydration in ethanol of different concentrations: 50%, 70%, 90%,
178 95%, and 100% (3x, 15 min/step) and then the slide(s) were subjected to critical point drying (CPD)
179 or washed 2x for 5 min with HMDS (Hexamethyldisilazane, Sigma-Aldrich). Silver paint or double-
180 sticky tape was used to mount on stubs which were then coated with gold-palladium (Au-Pd) and
181 either visualized, or stored in a dust-free dry area (desiccator) to view later. The visualization of the
182 biofilms was done using a FEI Quanta 600 FEG scanning electron microscope (SEM) at the Oklahoma
183 State University Electron Microscopy Core Facility.

184 *2.5 Statistical analysis.*

185 Each trial was performed in triplicate replication. All data were presented as the mean of
186 triplicate replications and standard deviation of the mean were represented by error bars. Statistical
187 analysis was done by using one way analysis of variance (ANOVA) and Holm-Sidak test for pairwise
188 multiple comparisons to determine significant differences ($p < 0.05$).

189 **3. Results**

190 *3.1. Choice of fluorescent substrate.*

191 We previously demonstrated that the mixed isomer, 5,6-carboxyfluorescein diacetate (5,6-
192 CFDA) was superior to the single isomer 5-carboxyfluorescein diacetate (5-CFDA) for intensity of
193 staining biofilms of *L. monocytogenes* [14] and included it again in this study for comparison of 5,6-
194 CFDA, 5,6-CFDA,SE and 5-CFDA. We obtained lower levels of fluorescence using 5,6-CFDA,SE
195 compared to fluorescence obtained with 5,6-CFDA (Fig. 1A). The 5,6-CFDA fluorophore was also
196 examined for whether the level used was limiting with the high levels of bacterial cells that we
197 observed during enzymatic detachment. When 2-fold dilutions of planktonic bacteria (from ~1 x 10^9
198 cfu/ml) were incubated with the same amount of 5,6-CFDA as used in adherence assays, no loss of
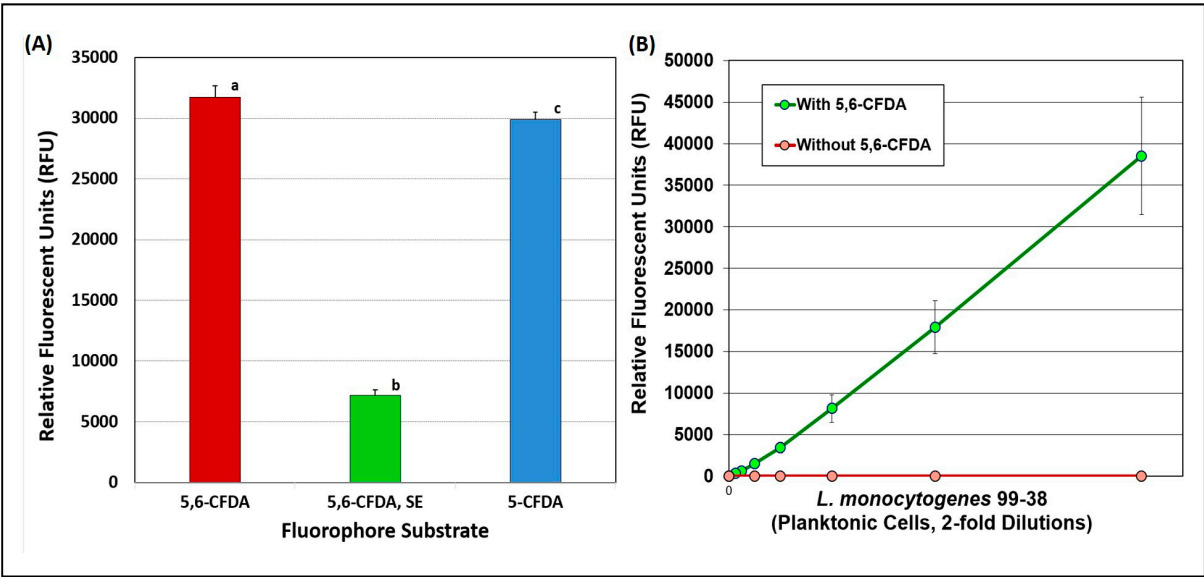199 signal linearity was observed (Fig. 1B).

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 lowercase letters are significantly different ($P < 0.05$). (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. Buffer washes with microplate biofilms.

Extended biofilms (3+ days of repeated media changes) of *L. monocytogenes* were examined for number of washes required to remove 'loosely retained' cells prior to enzymatic treatment for biofilm enumeration. When subjected to 4 rounds of buffer washes with automated 'shaking step' with each one, 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 (Fig. 2).
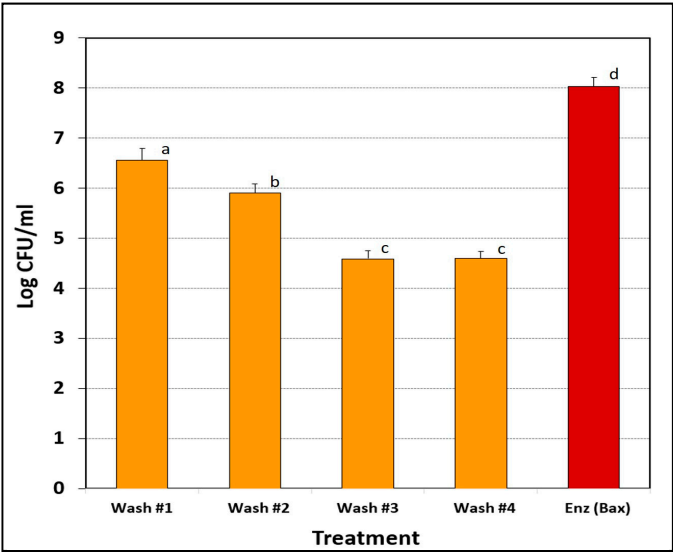

Figure 2. Enumeration of viable cells after multiple buffer washes with 0.05 M Tris buffer (pH 7.4) and after Bax protease treatment (after final wash) of *L. monocytogenes* 99-38 microplate biofilms. Data are presented as the mean of triplicate replications and error bars represent the standard deviation from the mean. Means with different lowercase letters are

218   significantly different ($p < 0.05$); means with the same lowercase letter are not significantly
219   different ($p > 0.05$).

220   *3.2. Screening of Listeria monocytogenes, Salmonella, and E. coli O157:H7 via fluorescence microplate assay.*

221   Bacterial strains were evaluated by previously established conditions for the fluorescence
222   microplate assay [14]. The adherence characteristics of numerous strains of *L. monocytogenes* isolated
223   from ready-to-eat meat processing facilities has already been examined [20]. In this study, we again
224   confirmed that *Listeria monocytogenes* 99-38 was a strongly-adherent strain in comparison with a
225   'weakly-adherent' *L. monocytogenes* CW35 (Fig. 3A).
226   Likewise, a fluorescence microplate assay of various serovars of *Salmonella* from our culture
227   collection demonstrated that *Salmonella* Montevideo FSIS 051 was the most adherent strain and could
228   be useful in development of microplate biofilms for testing of various antimicrobial interventions in
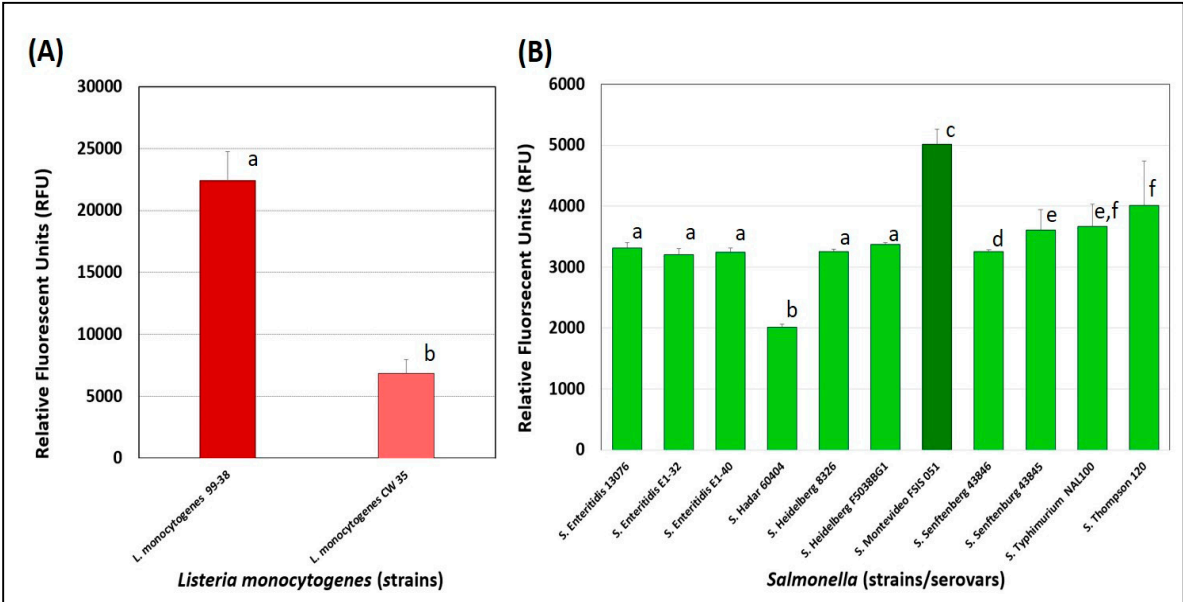229   a convenient microplate format (Fig. 3B).

230   **Figure 3.** Comparison of adherence of (a) *Listeria monocytogenesd* and (b) *Salmonella* by
231   microplate fluorescence assay with 5,6-CFDA. Data are presented as the mean of triplicate
232   replications and error bars represent the standard deviation from the mean. Means with
233   different lowercase letters are significantly different ($p < 0.05$); means with the same
234   lowercase letter are not significantly different ($p > 0.05$).
235
236   We also examined a variety of *E. coli* O157:H7 strains in our collection that were isolated from
237   cattle and cattle facilities along with a known biofilm forming *E. coli* O157:H7 strain (F-4546) to screen
238   for strong adherence as an indication of potent biofilm formation using the fluorescent adherence
239   assay (Fig. 4). Although some strains were moderately adherent (K-3995, K-4492), none were as
240   strongly adherent as the *E. coli* O157:H7 F-4546 control strain that is known for adherence (Fig. 4)
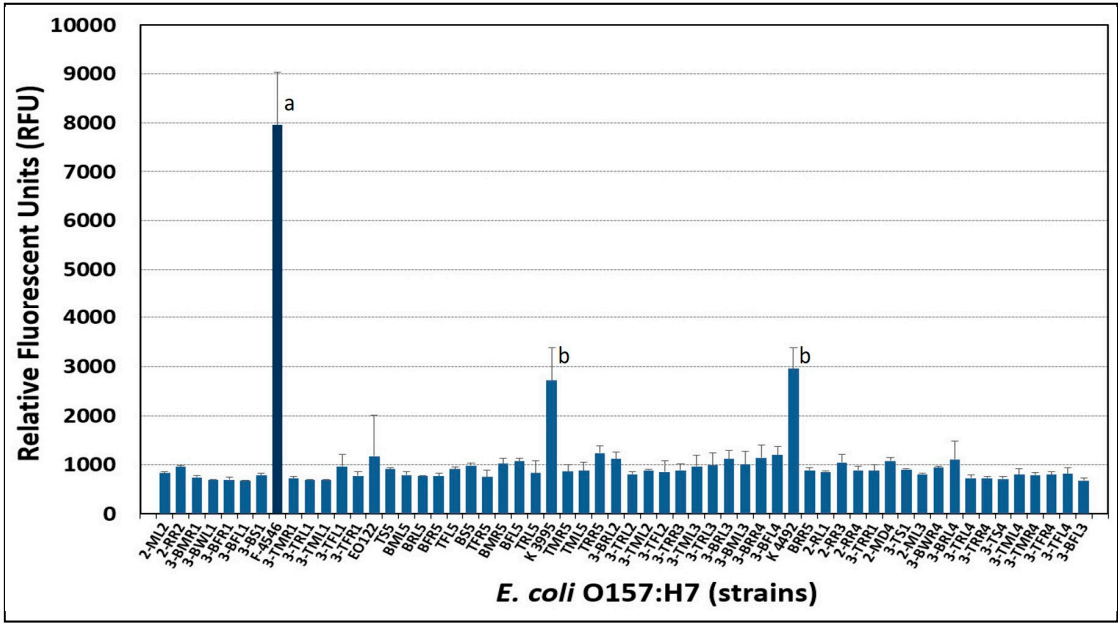
Figure 4. Comparison of adherence of various strains of *E. coli* O157:H7 by microplate fluorescence assay with 5,6-CFDA. Data are presented as the mean of triplicate replications and error bars represent the standard deviation from the mean. Means with different lowercase letters are significantly different ($p < 0.05$); means with the same lowercase letter are not significantly different ($p > 0.05$).
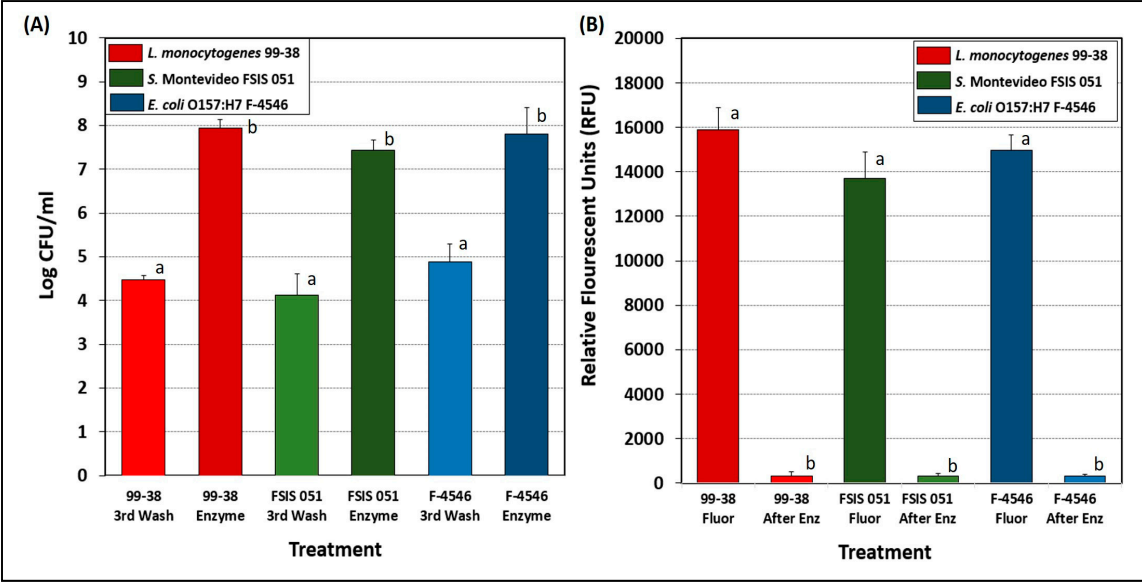

Figure 5. Comparison of enumeration and fluorescence data with *L. monocytogenes* 99-38, *Salmonella* Montevideo FSIS 051, and *E. coli* F-4546, before and after enzyme treatment of microplate biofilms. (A) Cell enumeration after 3rd round wash buffer followed by Bax protease release of adhered cells 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 triplicate replications and error bars represent the standard deviation from the mean. Significant differences of data from treatments with the same strain; data with different lowercase letters are significantly different ($p < 0.05$).

256   *3.3. Development of expanded biofilms in microplates.*

257   In previous studies, we initially used a short 2-3 day repeated incubation in microplates to
258   establish differences in the abilities of different strains to adhere to microplate surfaces [14]. In this
259   study, we were preparing to establish conditions for a robust biofilm for use in subsequent studies.
260   This was done by repeated cycles of growth, adherence, washing away of planktonic/loose cells, and
261   addition of sterile media to continue growth from those cells attached to microplate surfaces. Using
262   the pronase E to recover and enumerate attached bacterial cells from microplates similarly as
263   described previously with Bax protease [14]. The data shows that we were able to incrementally
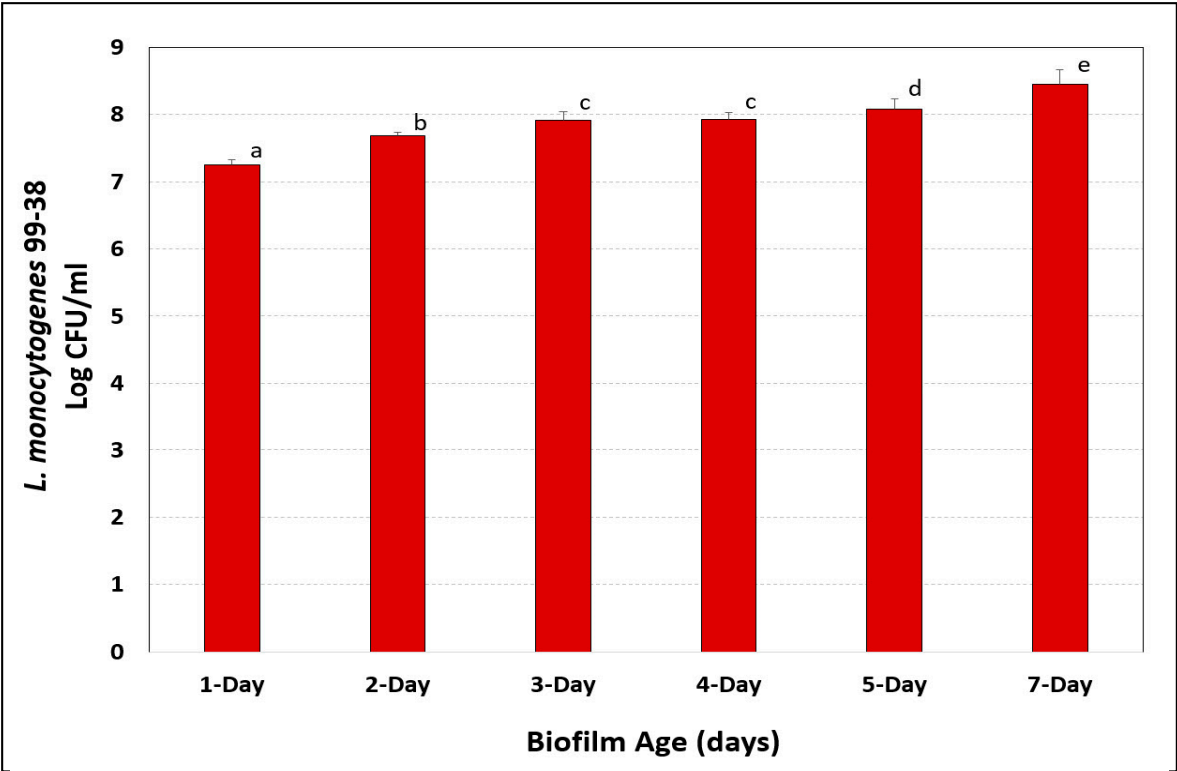264   increased the level of adhered cells by >12-fold over 7-days (Fig. 6).

265   **Figure 6.** Enumeration of *L. monocytogenes* 99-38 biofilm levels over time after repeated
266   incubation in microplates. Planktonic cells were removed daily, washed with buffer, and
267   replaced with fresh sterile media. Attached cells were enumerated by detachment with
268   pronase E and represented as the means of triplicate replications; error bars represent the
269   standard deviation of the means. Bars with different lowercase letters represents significant
270   differences in the means ($p < 0.05$); bars with the same lowercase letters are not significantly
271   different ($p > 0.05$).

272   *3.4. Evaluation of various enzymes for bacterial detachment and enumeration.*

273   In prior assays, we have used both Bax protease [14] and pronase E (Fig. 6) for enumerating
274   detached cells from surfaces. As we were trying to establish a set protocol that would be used to
275   examine antimicrobials against biofilms of multiple pathogens, we decided to re-evaluate various
276   enzymes to identify one that would be more suitable for all 3 pathogens that we have been working
277   with to treat and enumerate bacterial cells in extended biofilms.
278   Although all the enzymes tested worked to obtain high level counts from 7-day biofilms, three
279   were nearly equal for both *L. monocytogenes* 99-38 and *E. coli* O157:H7 F4546, including Bax protease,
280   pronase E, and trypsin (Fig. 7). However, for *Salmonella*, there was a clear difference between trypsin
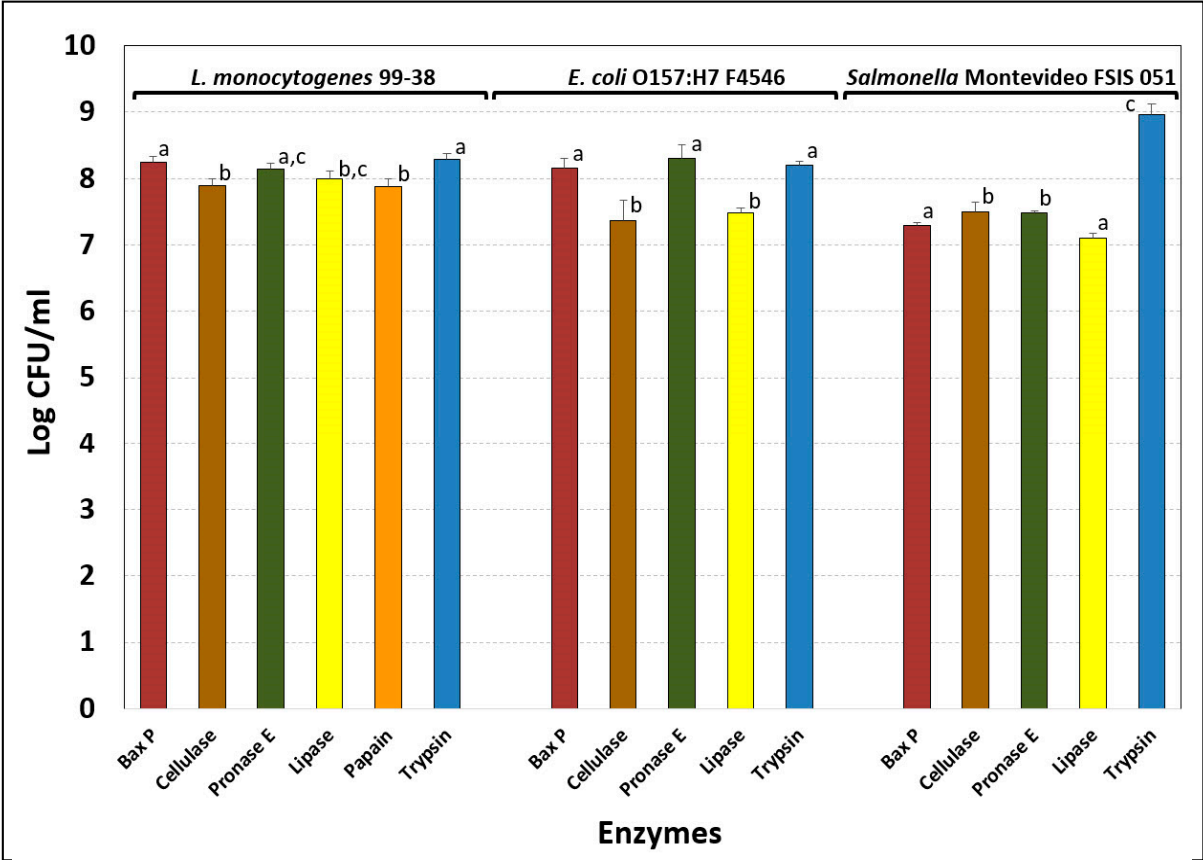281   and the other enzymes (Fig. 7).

282   **Figure 7.** End point enumeration of *L. monocytogenes* 99-38 biofilm levels detached after
283   treatment with various enzymes; Bax protease, cellulase, pronase E, papain, trypsin, or lipase.
284   Planktonic cells were washed and replaced daily with fresh sterile media for up to 7 days. The
285   levels of detachment are represented in terms of average log CFU/ml; data bars represent the means
286   of triplicate replications and error bars represent standard deviation of the means. Data bars with
287   different lowercase letters represent significant differences in the means ($p < 0.05$); data bars with
288   the same lowercase letters are not significantly different ($p > 0.05$).

289   The 7-day biofilms for *L. monocytogenes* 99-38, *E. coli* F4546, and *Salmonella* Montevideo FSIS 051
290   were also visibly different (Fig. 8). The *Listeria* looked like clean, naked hotdogs (Fig. 8A) while the
291   *E. coli* (Fig. 8B) and *Salmonella* (Fig. 8C) appeared to be coated with a film. Each of these biofilms
292   provided >8 log CFU/ml in 200-300 ul when recovered from microplate wells (with trypsin) and
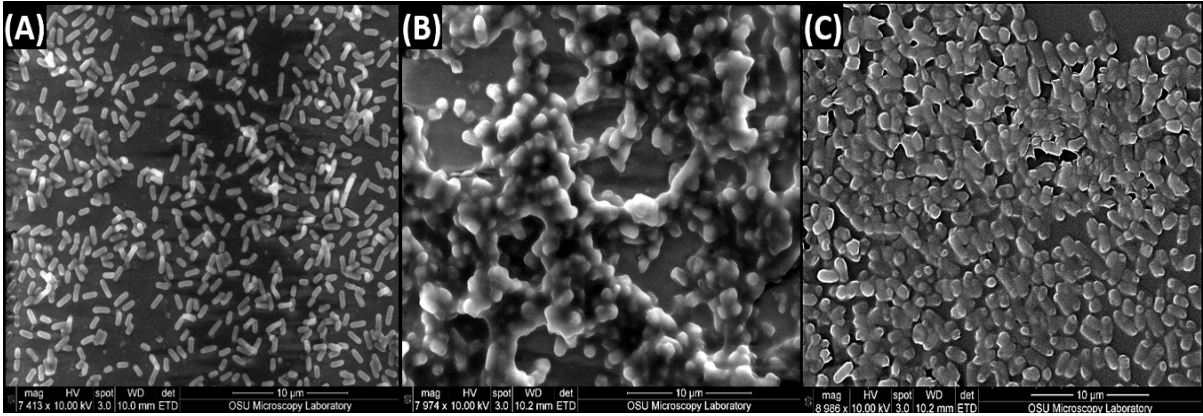293   enumerated on petri plates (Fig. 7).

294   **Figure 8.** Scanning electron microscopy (SEM) of extended 7-day biofilms prepared on
295   slide chambers from (A) *Listeria monocytogenes* 99-38, (B) *E. coli* O157:H7 F4546, and (C)
296   *Salmonella* Montevideo FSIS 051.

4. Discussion

Biofilm growth has been measured in different ways, from plating cells collected from surface swabs to staining of biofilms with crystal violet. Biofilms in microplates have been stained with crystal violet and absorbance measurements taken from dye recovered in destaining solutions [21,22], or biofilms stained with crystal violet have been measured directly in microplates with a plate reader [23], to vortexing with glass beads to recover cells for enumeration [24] or sonication after enzyme treatment to dislodge cells [25]. Microplate adherence and fluorescence visualization was examined to mimic flow cytometry where individual cells were 'counted' based on internalized fluorophores; we presumed that the method could be exploited for a qualitative assessment of adherence by cells attached *in situ* to microplate wells and using a fluorescence reader.

In prior and current studies to select the strongest adhering strains, we chose to use 'non-treated' microplates in order to assess the bacterial strains' inherent and unaided ability to adhere (i.e., 'treated' are often used with tissue culture assays to promote adherence). Although we continued to use the non-treated microplates in the development of biofilms, it is possible 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 into the plates and/or leave significant amounts of wash fluid behind.

Fluorescein is a fluorescing compound that can freely diffuse through cell membranes. It's use became popularized by applications in flow cytometry visualization [26], vital staining of live/dead bacterial cells [27], and applications allowing visualization by microscopy and studies in apoptosis [28]. It can also be quenched by modification with diacetate which provides an advantage in that only intracellular CFDA will fluoresce when it is hydrolyzed by esterases in the cytoplasm of bacterial cells, causing a significant spike in intracellular fluorescence. Unmodified fluorescein can also fluoresce externally to the cells. Therefore it is unnecessary to wash extracellular 5,6-CFDA away from treated cells as it doesn't fluoresce (but we do it anyway). The purported benefit of the succinyl ester of 5,6-CFDA (5,6-CFDA,SE) was that in addition to the diacetate modification, the -SE modification would allow it to be retained longer intracellularly because of the propensity to bind to amino groups. Fuller et al. [29] 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. This feature may have not have been useful in our application as measurements were always made immediately after incubation with fluorophore substrate, however prior data showed that fluorescence levels change over time and we wondered whether this modification would stabilize intracellular fluorescence levels to achieve higher and more sensitive detection levels. We found that 5,6-CFDA not only performed better than 5-CFDA or 5,6-CFDA,SE (Fig. 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 degree of adherence of cells as in a biofilm. 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 while using the same amount of fluorophore substrate suggesting that the levels used in our biofilm assays were not limiting (Fig. 1B). The fact that biofilm adhered cells are likely diffusion limited compared to planktonic cells further suggests that levels of fluorophore are not limiting in our biofilm assays.

Microplate biofilms comprised of *L. monocytogenes* 99-38 were washed multiple times with buffer before enzymatic treatment in order to remove the final media containing bacterial broth culture and any loosely adhered cells so that the resulting enzymatic enumerations would be more representative of the biofilm population. The plate washer had a built-in shake mode that provided a standardized shaking regimen between washes that helped to release loosely held cells attached directly to the substrate surface or to other cells. We proceeded with washes until the level of planktonic cells recovered in buffer stabilized as a measure of a sufficient degree of washing as there is likely to always be a release of some proportion of cells from the biofilm (Fig. 2).

In establishing conditions for *Listeria*, *Salmonella*, and *E. coli* biofilms, we had previously performed a robust screening of numerous strains of *L. monocytogenes* and again confirmed *L.*

349   *monocytogenes* 99-38 [14] as a robust, strongly-adherent strain that would serve well in the formation
350   of biofilms in this study (Fig. 3A). We further screened strains of *E. coli* O157:H7 and *Salmonella* in
351   our collection that could also be representative of good biofilm formers in preparation of a project to
352   evaluate sanitizers on biofilms of *Listeria, Salmonella,* and *E. coli* O157:H7. Among the *Salmonella*
353   strains/serovars in our collection, *Salmonella* Montevideo FSIS 051 was significantly more adherent
354   than the other strains tested (Fig. 3B). *E. coli* F-4546, a control strain implicated in biofilm formation
355   [30], was also the most adherent from among the 58 strains of *E. coli* O157:H7 tested (Fig. 4). Based
356   on the fluorescent microplate data with these strains, we continued optimization of biofilm adherence
357   with *L. monocytogenes* 99-38, *Salmonella* Montevideo FSIS 051, and *E. coli* F-4546 (Figs. 3 and 4).
358   The wash parameter was examined for all 3 of the selected strains (99-39, FSIS 051, and F-4546)
359   to insure that significantly lower levels were in the final wash than were when recovered after
360   protease treatment (Fig. 5A). Although seemingly high levels of cells are still recovered in the wash
361   buffer, they represent a small proportion relative to the levels attached to the well surfaces (Fig. 5A).
362   Similar 'before and after' trials using fluorescence assays were performed with a full biofilm load and
363   compared to similar wells after detachment of cells with protease, washing with buffer, and
364   application of fluorescent substrate (Fig. 5B). The absence of significant detectable signal after enzyme
365   treatment supports the data obtained with enumeration after enzymatic detachment. Further, since
366   application of 5,6-CFDA did not affect viability of cells, wells treated with the fluorophore and
367   examined on the plate reader could be subsequently treated with protease and plated so that plate
368   counts could be obtained for the same wells used for fluorescence measurements.
369   At a recent food safety meeting, data was presented with 1-24 hr surface-attached cells defined
370   as biofilms, resulting in an ensuing discussion on "what degree of surface attachment and growth
371   constitutes a biofilm"? In preparation for an upcoming study that would assess the effect of sanitizers
372   on biofilms, we decided to increase the robustness of our biofilms by starting with a dilution of cells
373   in media that would initiate growth with repeated cycling of buffer wash, fresh media, incubation,
374   and growth, repeated daily for 7 days. Microplate biofilms were examined in 1-to-7 day increments
375   (i.e., different plates for the respective days) by enzymatic detachment and enumeration of cell levels
376   to insure that incremental increases were occurring during the extended cycle times (Fig. 6). It was
377   also good to re-examine the enzymes that could be used to detach bacterial cells for all 3 organisms
378   used in our repertoire for microplate biofilm assays since we did not previously examine biofilms
379   from *E. coli* or *Salmonella*. Using a variety of enzymes, Bax protease, pronase E, and trypsin
380   enumerated comparable levels of detached cells for *L. monocytogenes* 99-38 and *E. coli* F-4546 (Fig. 7).
381   However, trypsin was significantly better in providing higher counts than the other enzymes with
382   *Salmonella* Montevideo FSIS 051 and therefore trypsin was the enzyme of choice going forward (Fig.
383   7). Trypsin was a reasonable choice because it has been long been used in tissue culture studies for
384   releasing tissue culture cells from flasks [31] and further, it is the least expensive of the enzymes used
385   in this study, and is often sold in a convenient liquid form.
386   SEM examination of 7-day biofilms found visual differences between them (Fig. 8). After 7-days
387   of recycling media addition, incubation, buffer washes, fresh media, and repeat, SEM analysis of *L.*
388   *monocytogenes* 99-38 biofilms looked like clean cells attached to a surface (Fig. 8A) whereby *S.*
389   Montevideo FSIS 051 (Fig. 8B) and *E. coli* F-4546 (Fig. 8C) appear as a matt of overlapping cells with
390   a 'coating' on the bacterial cells. This difference could be due to the presence of extracellular
391   polysaccharides that Gram-negative bacteria are known to produce [32].

5. Conclusions

393   We feel that we have improved our prior microplate biofilm assay that includes fluorescence
394   analysis for qualitative assessment of cellular attachment and enzymatic detachment for
395   quantification of cell enumeration. For the bacteria we have chosen, 5,6-CFDA and trypsin are both
396   the most effective and least expensive of the alternative components we examined. Bacteria continue
397   to leach from such biofilms, as shown in our buffer wash study, and mimics similar situations in food
398   processing facilities where potential contact surface biofilms may contaminate passing foods as foci
399   of contamination. We hope to examine the effect of sanitizers on these extended biofilms that would

400 reflect a potential problem in food processing plants where sanitation may be lacking or insufficiently
401 applied, resulting in the accumulation of pathogenic biofilms. It will be interesting to see if the
402 observation of potential EPS coating of *Salmonella* and *E. coli* biofilms affects sanitizer lethality on
403 biofilms related to these organisms.

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